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Variation of phytosterols and steryl ferulates in wheat grains and fractions

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ACADEMIC DISSERTATION

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ABSTRACT

Phytosterols are plant-derived bioactive compounds known for their serum cholesterol-lowering ability and other health benefits. Phytosterols may occur as free sterols or various conjugates, e.g. ferulic acid esters. Cereals are an important source of natural phytosterols and sterol conjugates. However, the variation in occurrence and distribution of these compounds in cereal grains has not been adequately studied. As a part of the EU FP6 project HEALTHGRAIN, the effects of genotype, environment and dry fractionation on phytosterol and steryl ferulate contents in wheat kernels were studied.

Wheat genotypes were grown at one location in Hungary over three consecutive years (2005 to 2007) and at three additional locations in Europe (Poland, France and the United Kingdom) in 2007. In 2005, 175 wheat lines were included in the diversity screen trial, and selected lines were further studied for environmental variation, focusing on bread wheat. Variation within wheat grains was studied by analysing wheat and bran fractions produced by novel and conventional dry fractionation processes. All genotypes and fractions were analysed for phytosterols using GC-FID and selected ones for steryl ferulates using HPLC-UV.

The contents and compositions of sterol compounds in wheat were significantly affected by the genetic factors and growing location, whereas no considerable year-to-year variation was observed. The highest phytosterol contents were observed in ancient einkorn wheat and durum wheat genotypes and the lowest in bread wheat lines. Bread wheat contained 670–959 µg/g DM phytosterols, of which 7–9% occurred as steryl ferulate conjugates (79–123 µg/g DM), when grown at a single location during one year. Within various environments, the highest levels of sterol compounds were observed in genotypes cultivated in Hungary, and the lowest in those cultivated in the UK and France. Sitosterol was the main sterol species and the widest variation was observed in the stanol contents. Stanol species were the main compounds in the steryl ferulate fraction. Small kernels with a high bran content contained higher levels of sterol compounds than large ones. The contents and compositions of wheat fractions varied substantially, with the total contents of phytosterols and steryl ferulates ranging 6- and 120-fold, respectively. Phytosterols and steryl ferulates were concentrated in the bran. Within the bran layers, phytosterols were accumulated in the intracellular contents of the aleurone layer and intermediate layers, whereas steryl ferulates accumulated in the intermediate layers.

Knowledge of the natural variation of phytosterol compounds in wheat enables the selection of wheat varieties and fractions with high and stable phytochemical content. Exploitation of such wheat grains or fractions in cereal foods would increase the intake of bioactive compounds and enhance public well-being and health in a natural way.

PREFACE

I found my way into the world of phytosterols in 2001 during my master studies in the Division of Food Chemistry at the Department of Food and Environmental Sciences, University of Helsinki. There was no turning back and I immersed myself in this research during 2006-2012. The study was funded by the European Commission in the Communities 6th Framework Programme project HEALTHGRAIN (FP6-514008) and The Finnish Graduate School on Applied Bioscience: Bioengineering, Food & Nutrition, Environment (ABS Graduate School), which are acknowledged for the financial support. I also thank Raisio Research Foundation for a scholarship.

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I warmly thank my co-authors Associate Professor Laura Nyström, Dr Xavier Rouau, Dr Youna Hemery and MSc Minnamari Edelmann for comments and conversations during the preparation of the manuscripts. I also appreciate the help provided by the project partners in Hungary, Dr Zoltan Bedõ and MSc Mariann Rakszeki. Our HEALTHGRAIN research team in Viikki is thanked for the seamless cooperation and enthusiastic attitude during the project. MSc Susanna Heikkinen, MSc Kirsti Herttua, MSc Johanna Juntunen, MSc Tuuli Koivumäki, MSc Milla Nurmi and Dr Karin Struijs are sincerely thanked for collaboration and assistance in the laboratory.

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I want to thank all of my present and former colleagues in Viikki. I have been fortunate to get to know so many great people during these years, and it has been a pleasure working with you. Minttu and Susu, with you I have been able to talk about

everything between heaven and earth (mainly about the kids, of course) and I am grateful for your friendship and support. I am thankful to Tuuli and Petri for their patience with my endless questions about "computer things". Special thanks to Mari for sharing thoughts and laughs in our workroom during the recent years, while struggling towards the same goal. I also remember with warmth the “old” sterol girls, who created such a pleasant working athmosphere and aroused my enthusiasm for further research.

I owe my deepest thanks to my dear family, relatives and friends for always being there for me. Especially I thank my brother Jan for support and caring as long as I can remember. Without you I possibly would not have found my way to Viikki in the first place.

Finally, from the bottom of my heart, I want to thank my husband Moritz for love, encouragement and believing in me, and our little sweethearts Sofia and Ida for the happy, joyful moments together and for giving me a perspective. You have made this journey unforgettable. These years with you by my side have been the best time of my life. With all the ups and downs, I would not change a thing!

Helsinki, October 2012

Tanja Nurmi

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which are referred to in the text by the Roman numerals **I-IV**:

I Nurmi T, Nyström L, Edelmann M, Lampi A-M, Piironen V. 2008. Phytosterols in wheat genotypes in the HEALTHGRAIN diversity screen. J Agric Food Chem 56:9710-5.

II Nurmi T, Lampi A-M, Nyström L, Piironen V. 2010. Effects of environment and genotype on phytosterols in wheat in the HEALTHGRAIN diversity screen. J Agric Food Chem 58:9314-23.

III Nurmi T, Lampi A-M, Nyström L, Turunen M, Piironen V. 2010. Effects of genotype and environment on steryl ferulates in wheat and rye in the HEALTHGRAIN diversity screen. J Agric Food Chem 58:9332-40.

IV Nurmi T, Lampi A-M, Nyström L, Hemery Y, Rouau X, Piironen V. 2012. Distribution and composition of phytosterols and steryl ferulates in wheat grain and bran fractions. J Cereal Sci 56:379-88.

Contribution of the author to papers I to IV:

I Tanja Nurmi planned the study together with the other authors, and she performed part of the experiments. She had the main responsibility for interpreting the results, and she was the corresponding author of the paper.

II-IV Tanja Nurmi planned the study together with the other authors. She had the main responsibility for the experimental work and interpreting the results, and she was the corresponding author of the paper.

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ABBREVIATIONS

ANOVA	analysis of variance
BSTFA	N,O-bis(trimethylsilyl) trifluoroacetamide
CAF	cycloartenyl ferulate
CHD	coronary heart disease
CV	coefficient of variation
DHC	dihydrocholesterol
DM	dry matter
DUS	distinctness, uniformity and stability
EFSA	European Food Safety Authority
FID	flame ionisation detector
FW	fresh weight
GC	gas chromatography
HCl	hydrochloric acid
HPLC	high-performance liquid chromatography
INRA	French National Institute for Agricultural Research
KOH	potassium hydroxide
LDL	low density lipoprotein
LSD	least significant difference
MS	mass spectrometry
NCEP	National Cholesterol Education Program
PC	principal component
PCA	principal component analysis
r	Pearson's correlation coefficient
SD	standard deviation
SIM	selective ion monitoring
SiOH	silica
SPE	solid phase extraction
TKW	thousand kernel weight
TLC	thin-layer chromatography
TMCS	trimethylchlorosilane
TMS	trimethyl silyl
UPOV	the International Union for the Protection of New Varieties of Plants
UV	ultraviolet

CONTENTS

1 INTRODUCTION

The cultivation of wheat started about 10 000 years ago during the establishment of agriculture. The earliest wheat species grown were diploid einkorn and tetraploid emmer wheats, which are the progenitors of all wheat types cultivated today. Nowadays, wheat is, together with rice and corn, among the major cereal crops in the world, with a global production of 650 million tonnes annually (FAOSTAT 2012). Wheat also is the most popular cereal type cultivated and consumed within Europe, the yearly production being approximately 200 million tonnes. Cultivation of wheat is almost entirely concentrated on common hexaploid bread wheat, although other wheat species, such as spelt, durum wheat and ancient wheat types, are grown to a lesser extent in certain regions (Shewry 2009a).

Whole grains are defined as consisting of intact, ground, cracked or flaked kernel after the removal of inedible parts, the principal anatomical components being present in the same ratio as in the intact kernel (Asp et al. 2010; Van der Kamp 2012). The consumption of wholegrain cereals is thought to contribute to several health benefits, since they are known to be protective against chronic diseases related to metabolic syndrome, including obesity, type 2 diabetes and cardiovascular disease. Whole grains contain dietary fibre, micronutrients and numerous phytochemicals, which are thought to be responsible for their beneficial health effects (Liu 2007; Fardet 2010). The European Union 6th Framework Programme integrated project HEALTHGRAIN (2005 to 2010) was initiated to find the ways to increase the intake of health protective components from wholegrain cereals, namely bread wheat, durum wheat, spelt, emmer wheat, einkorn wheat, rye, oat and barley (Poutanen et al. 2008; Ward et al. 2008). The focus was on bread wheat and bioactive compounds, such as dietary fibre components, phytosterols, tocols, folate, alkylresorcinols and phenolic acids. The natural variations of the compounds, as well as the technological aspects, were investigated during the project.

Cereals are an important natural source of phytosterols, plant-derived bioactive components. Phytosterols are found as free sterols or various conjugates. The ferulic acid conjugates of phytosterols, i.e. steryl ferulates, only occur in certain cereals, such as rice, corn and wheat (Mandak and Nyström 2012a). Phytosterol compounds are particularly known for their ability to lower the serum total and low-density lipoprotein (LDL) cholesterol values by reducing the absorption of dietary and biliary cholesterol in the small intestine (Trautwein et al. 2003). In addition, phytosterols may prevent cancer and inhibit inflammation (Berger et al. 2004; Jones and AbuMweis 2009; Othman and Moghadasian 2011). Steryl ferulates also possess antioxidant properties (Nyström et al. 2005). Due to the

health-promoting effects, phytosterols are often added to foods, such as spreads or yoghurt, to produce functional foods. However, the beneficial effects can also be achieved with moderately low doses obtained from natural sources as part of the normal daily diet (Ellegård et al. 2007). At the same time, there is a growing demand for naturalness amongst consumers (Mellentin 2012). Therefore, cereal products may provide as a good natural alternative to enriched foods as source of phytosterol compounds. To be able to increase the intake of phytosterols and other bioactive components from cereals and particularly from wheat products, knowledge of their natural variation is required. However, the genetic and environmental variation of sterols and steryl ferulates in wheat has not been adequately studied.

Like many other phytochemicals, phytosterols are suggested to concentrate in the bran and germ of the wheat grain, whereas steryl ferulates accumulate only in the bran layers (Nyström et al. 2007). The further distribution of sterol compounds in the various layers of the bran is not known. Furthermore, bran is regarded as a by-product in the common wheat milling process, and the refining of the wheat thus results in losses of the bioactive compounds. The utilisation of phytosterol-rich bran fractions in foods would increase the intake of these compounds from natural sources.

In the present study, the variation in the content and composition of wheat phytosterols and steryl ferulates caused by genetic and environmental factors was examined. Furthermore, the distribution of sterol compounds within the wheat grains was studied. The literature review gives an overview of the structural features, potential health effects and sources of phytosterols and steryl ferulates. The occurrence and diversity of these compounds in wheat grains and fractions are reviewed. The experimental section summarises the data presented in the attached papers **I-IV**, in which the effects of genotype, environment and dry fractionation on phytosterols and steryl ferulates in wheat are studied. Finally, the results are comprehensively discussed, and the concluding remarks are made.

2 REVIEW OF THE LITERATURE

2.1 Phytosterols and steryl ferulates

2.1.1 Structure

Phytosterols

Phytosterols (i.e., plant sterols) are steroid alcohols belonging to the triterpene family. These phytochemicals are synthesised from mevalonate via a complex enzymatic pathway. Mevalonate is first converted to squalene and then further to cycloartenol and other sterol compounds (Piironen et al. 2000; Schaller 2003).

The basic structure of phytosterols is similar to that of cholesterol found in animals. They are composed of a tetracyclic cyclopenta[*a*]phenantrene ring and a side chain attached to C-17 (Figure 1). Sterols may have a double bond between C-5 and C-6 (Δ^5 phytosterols) or between C-7 and C-8 (Δ^7 phytosterols). Stanols are saturated forms having no double bonds within the ring structure. In this thesis, the word "phytosterol" is used to describe both unsaturated sterol and saturated stanol forms, if not otherwise stated. Based on the number of methyl groups in C-4, sterols are classified as desmethyl (none), 4-monomethyl (one methyl group) or 4,4'-dimethyl sterols (two methyl groups). Furthermore, the structure of the side chain may differ; C-24 may be attached by a methyl, ethyl or ethylidene group, and there may be a double bond, e.g., between C-22 and C-23 (Piironen et al. 2000; Moreau et al. 2002).

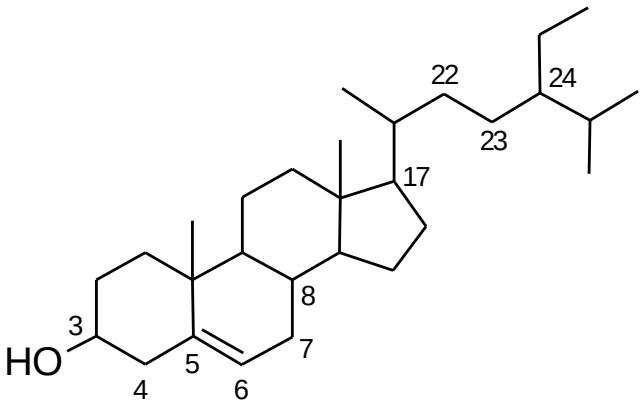

Figure 1. Chemical structure of a phytosterol (sitosterol is used as an example).

Phytosterols occur in plants as free alcohols and as various conjugates. In steryl glycosides and acylated steryl glycosides, the hydroxyl group in C-3 is attached with a glycosidic bond to a monosaccharide (e.g., glucose) or a fatty acid esterified monosaccharide, respectively. Fatty acid ester conjugates have a fatty acid bound with the hydroxyl group. In addition, phytosterols may occur as hydroxycinnamic acid esters esterified with ferulic or *p*-coumaric acid.

The free sterols, steryl glycosides and acylated steryl glycosides are structural components of plant cell membranes; they may be concentrated in certain parts of plasma membrane and organelle membranes (Piironen et al. 2000; Moreau et al. 2002; Tjellström et al. 2010; Cacas et al. 2012). They may also regulate the fluidity and permeability of the intracellular phospholipid bilayers and in some membranes they may be more abundant than phospholipids. The glycosidic conjugates of sterols are synthesised in the cytosolic side of the plasma membrane, the glycosylation of membrane-bound free sterols resulting in alterations in the biophysical properties of the membrane (Grille et al. 2010; Cacas et al. 2012). Steryl fatty acid esters are storage and transport forms of phytosterols, possibly localised with triacylglycerols in the intracellular lipid droplets and spherosomes (Gondet et al. 1994). Phytosterols also have other potential functions in plant cells, e.g., serving as precursors or substrates for other steroid compounds, such as brassinosteroids and steroidal saponins (Piironen et al. 2000).

Steryl ferulates

Steryl ferulates are phytosterol conjugates, which have a ferulic acid esterified to the hydroxyl group of the sterol or stanol (Figure 2). In rice, steryl ferulates are called γ-oryzanol. When not otherwise stated, steryl ferulate refers to both steryl and stanyl conjugates throughout this thesis. Various steryl ferulates differ in the structure of sterol moiety. Due to the polar ferulic acid moiety, steryl ferulates are less lipid-soluble than the corresponding free sterols.

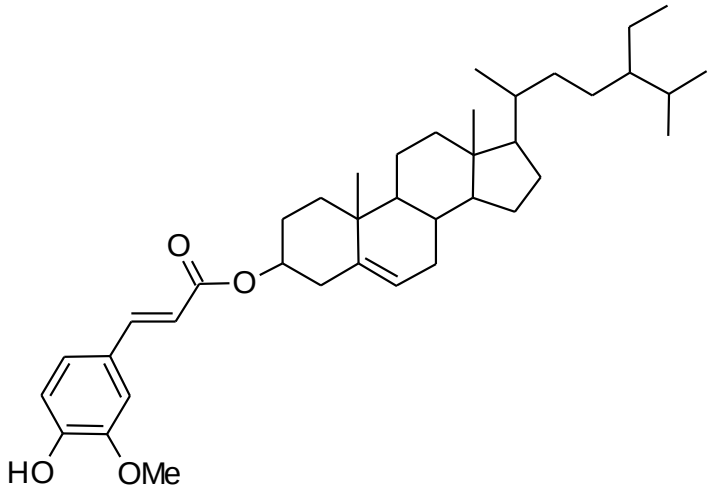

Figure 2. Chemical structure of steryl ferulate (sitosteryl ferulate is used as an example).

Steryl ferulates are uniquely found in certain grains and seeds. The biosynthetic pathway and localisation of steryl ferulates in the plant cells is not known; it remains to be elucidated, including where in the plant cells ferulation of sterols occurs and how it is regulated. Steryl ferulates may have a protective role in plant cells, since they were suggested to regulate microbial activity in the grains (Seitz 1989). Their function in plant cells may also be related to their antioxidant properties (Islam et al. 2009). Increased levels of steryl ferulates in brown rice grown in moderately elevated temperatures indicate involvement in plant stress responses (Britz et al. 2007).

2.1.2 Health-promoting effects

Phytosterols

Phytosterols have attracted much interest due to their health-promoting effects. The greatest attention has been paid especially to the cholesterol-lowering ability of phytosterols, which was first established in 1951 (Peterson 1951). Since the 1990s, increasing awareness of the potential health benefits of these phytochemicals has led to the production of functional foods enriched with free or fatty acid esterified phytosterols or stanols. The effect of sterols and stanols on lipid metabolism has been stated as one of the greatest discoveries in nutrition research in recent decades (Katan et al. 2009). In this section, the impact of phytosterols on serum cholesterol levels is reviewed in detail, and other possible biological properties are briefly discussed.

Serum cholesterol-lowering effect

Elevated cholesterol levels are a risk factor for coronary heart disease (CHD), and it has been estimated that reduction of cholesterol by 10% reduces cardiovascular death by 13% and total mortality by 10% (Gould et al. 1995). Law et al. (1994) assessed that a long-term reduction in serum cholesterol levels by 10% will lower the incidence of ischaemic heart disease by 50% at the age of 40, gradually decreasing to a 20% lower risk by the age of 70.

Phytosterols are known to decrease serum cholesterol levels by inhibiting dietary (exogenous) and biliary (endogenous) cholesterol absorption in the small intestine. Several potential mechanisms have been suggested to explain this effect (Trautwein et al. 2003; Jones and AbuMweis 2009). The most popular explanation is that phytosterols compete with cholesterol for incorporation into mixed micelles composed of bile salts and phospholipids (Figure 3). To be absorbed from the intestine into circulation, cholesterol must bind to these micelles. Because they have a higher affinity for the micelles than cholesterol, phytosterols displace the cholesterol in the micelles, resulting in a reduction of cholesterol absorption and subsequent reduction of serum low-density lipoprotein (LDL) and total cholesterol levels (Trautwein et al. 2003). Brown et al. (2010) previously suggested that the effect of phytosterol on the solubility of cholesterol into mixed micelles may vary depending on the structure of the sterol and fatty acid esterified into it. Phytosterols may also co-crystallise with cholesterol in the intestinal lumen forming nonabsorbable precipitates (Trautwein et al. 2003). In addition, phytosterols can possibly interfere with the enzymatic reactions needed for cholesterol absorption into the bloodstream. First, they may inhibit the hydrolysis of cholesterol esters to free cholesterol in the intestine, thus restraining cholesterol from merging into mixed micelles. Second, phytosterols may also suppress the esterification of free cholesterol occurring in enterocytes, which prevents its incorporation into chylomicrons. The absorption of phytosterols themselves is very low or negligible, and phytosterols are readily transferred from enterocytes back to the intestinal lumen by transporter proteins. One theory suggests that phytosterols increase the expression of certain transporter proteins and thus increase the excretion of cholesterol and phytosterols from the enterocytes to the lumen (Trautwein et al. 2003).

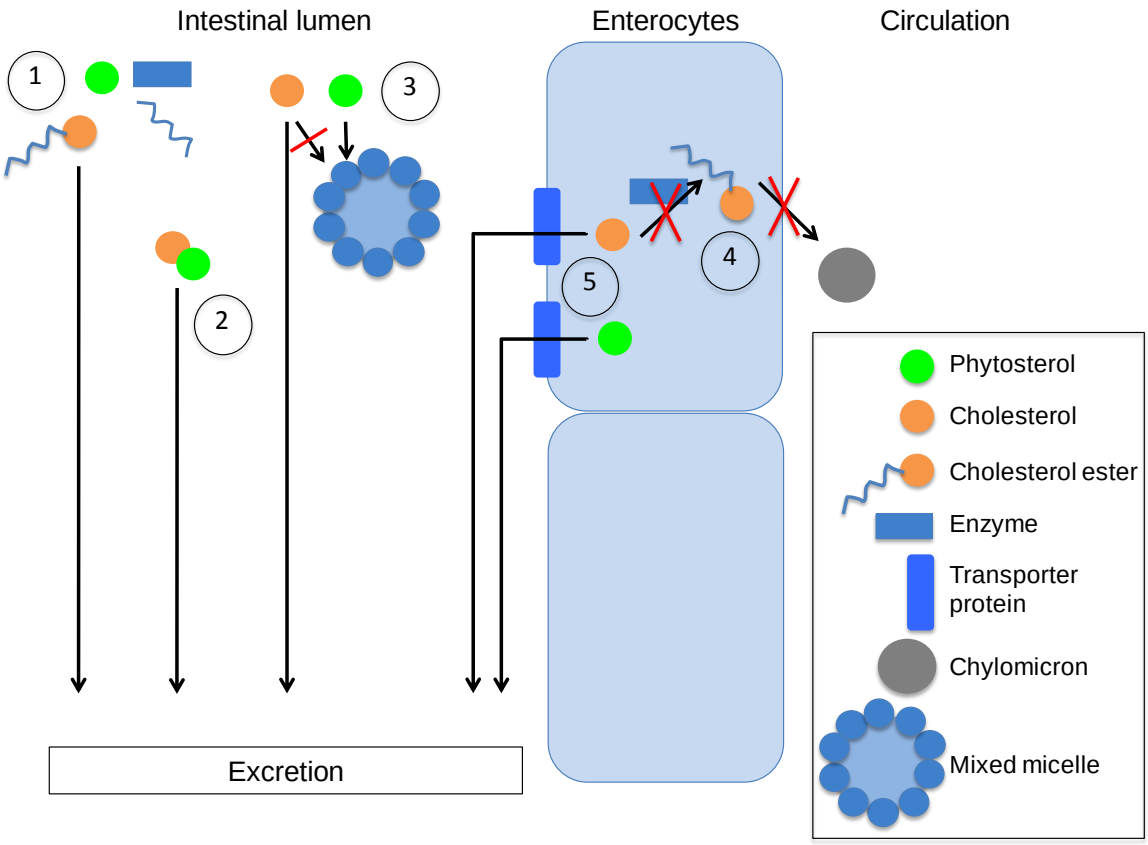

Figure 3. Proposed mechanisms for the cholesterol-lowering effect of phytosterols in the gastrointestinal tract. 1) Preventing the hydrolysation of cholesterol esters, 2) Co-crystallisation, 3) Competition of the micellar space, 4) Preventing the esterification of cholesterol and incorporation into chylomicrons and 5) Increasing excretion from enterocytes to the lumen by transporter proteins.

The effect of phytosterols on cholesterol metabolism has been extensively studied, and several meta-analyses have been published (Table 1). A previous meta-analysis summarised the data obtained in 59 clinical trials, and it was concluded that the consumption of foods containing phytosterols or stanols decreased LDL cholesterol levels by 0.31 mmol/l (AbuMweis et al. 2008). The effect was apparently dependent on the dose of phytosterols, frequency and time of intake, baseline LDL cholesterol levels and the food matrice. Based on 84 trials, a reduction of 0.34 mmol/l (8.8%) in the LDL cholesterol level was observed for a dose of 2.15 g/d phytosterols (Demonty et al. 2009). With a slightly higher phytosterol dose, approximately 15% and 11% decreases in serum LDL and total cholesterol levels, respectively, were reported for familial hypercholesterolemic subjects (Moruisi et al. 2006). Earlier, a meta-analysis of 41 trials showed that a daily intake of 2 g lowered the serum LDL cholesterol levels by 10% (Katan et al. 2003). An intake higher than 2.5 g/d resulted in little additional effects. A recent review also analysed the impact of high phytosterol intakes on cholesterol values and suggested a greater reduction on

cholesterol values with stanols compared to sterols at high doses (Musa-Veloso et al. 2011). This meta-analysis has, however, been criticised for making too far-reaching conclusions on the basis of the limited data available for high phytosterol intakes (Demonty et al. 2011). A few studies have proposed up to 17% reduction in LDL cholesterol level with an intake of approximately 9 g/d of free or esterified phytosterols or phytostanols (Gylling et al. 2010; Musa-Veloso et al. 2011).

Table 1. Meta-analyses and scientific opinions of the cholesterol-lowering effect of phytosterols and stanols.

Trials	Mean dose of sterols or stanols (g/d)	Reduction in LDL cholesterol	Reference
14	2	9–14%	Law 2000
41	2	10%	Katan et al. 2003
36	0.8–1.0	≥ 5%	Berger et al. 2004
4	2.3	10–15%	Moruisi et al. 2006
59	n.r.	0.31 mmol/l	AbuMweis et al. 2008
84	2.15	8.8%	Demonty et al. 2009
114	1.78–2.63	7.7–10.3%	Musa-Veloso et al. 2011
	1.5–2.4	7–10.5%	EFSA 2009
	3	11.3%	EFSA 2012a
	3	11.4%	EFSA 2012b

Based upon the cumulative knowledge on the cholesterol-lowering properties of phytosterols, the Panel on Dietetic Products, Nutrition and Allergies of European Food Safety Authority (EFSA) has stated that a daily phytosterol or stanol intake of 1.5–2.4 g tends to lower the serum LDL cholesterol level by 7 to 10.5%, which may reduce the risk of CHD (EFSA 2009). Recently, the EFSA (2012a; 2012b) further concluded that 3 g/d of phytosterols will decrease LDL cholesterol by 11.3 to 11.4%. A National Cholesterol Education Program (NCEP) expert panel has recommended the use of phytosterols or stanols (2 g/d) as part of a healthy diet and lifestyle to enhance the LDL cholesterol-lowering effects (NCEP 2001). The daily doses obtained from functional foods enriched with phytosterols are at the aforementioned level or even higher (Kuhlmann et al. 2005; Hearty et al. 2009). However, such a high intake of phytosterols can not be achieved from natural, non-enriched foods.

The cholesterol-lowering effect of phytosterols has also been observed with moderately low doses of phytosterols. However, when phytosterols are supplied as small amounts or as a part of the normal daily diet from natural sources, the impact of phytosterols on the

absorption of cholesterol or on serum cholesterol levels is a much less studied area than the effect obtained by enriched foods. The absorption of cholesterol was previously reduced by 12–28% in healthy subjects eating 150 to 300 mg corn oil phytosterols in the single test meals consumed twice at an interval of 2 weeks when compared to phytosterol-free corn oil (Ostlund et al. 2002). Similarly, the intake of 328 mg of wheat germ phytosterols in muffin, consumed 3 times at 2 weeks intervals, reduced the cholesterol absorption by 30% (Ostlund et al. 2003). Phytosterols were shown to be the cholesterol absorption-lowering component in corn oil and wheat germ. The ability of natural dietary phytosterols to inhibit cholesterol absorption was also observed in the recent study of Lin et al. (2010). Their subgroup of healthy subjects with a phytosterol-abundant diet and a mean intake of 512 mg/d had significantly reduced cholesterol absorption in comparison to the group with a phytosterol-poor diet and a mean intake of 140 mg/d. Earlier, an uncooked vegan diet with a high phytosterol content (intake approximately 900 mg/d) was shown to significantly lower the serum total and LDL-cholesterol levels in subjects with rheumatoid arthritis (n = 16), when compared to a control group with a normal daily diet (Ågren et al. 2001). Escurriol et al. (2009a) studied the effects of a Mediterranean diet supplemented with virgin olive oil or nuts on high cardiovascular risk subjects. A diet supplemented with nuts increased the phytosterol intake by 158 mg/d and decreased the LDL cholesterol values by 8.3% compared to the low-fat diet group (which had phytosterol intake increased by 15 mg/d), showing that even modest changes in the phytosterol intake can significantly affect the serum cholesterol levels.

The increased intake of natural phytosterols in normal daily diet was found to result in lower total and LDL cholesterol levels within free-living populations in a few studies. The first study included British subjects (n = 22 256) whose normal dietary intake of phytosterols ranged from 59 to 749 mg/d (Andersson et al. 2004). Total cholesterol was 2.4 to 4.1% lower and LDL cholesterol 3.0 to 3.5% lower in a subgroup with the highest mean intake (467 mg/d) compared to the subgroup with the lowest mean intake (177 mg/d) of natural phytosterols. The effect on cholesterol levels was higher among men than among women. Within a Swedish population (n = 77 652), men with the highest phytosterol intake (mean 327 mg/d) had 2.6% lower total and 3.1% lower LDL cholesterol levels than those with the lowest intake (185 mg/d) (Klingberg et al. 2008). The total and LDL cholesterol levels were 3.5% and 3.2% lower, respectively, within women with the highest mean intake (270 mg/d) compared to those with the lowest intake (160 mg/d). A recent study of Wang et al. (2012) amongst Chinese men and women (n = 3940) suggested 5.0 to 6.4% and 6.2 to 7.1% lower total and LDL cholesterol levels, respectively, in a subgroup with the highest phytosterol intake (447 mg/d) when compared to a subgroup with the

lowest intake (< 206 mg/d). The cholesterol levels were affected less within women than within men. Another study with even fewer participants (n = 85) from Spain also observed lower LDL cholesterol values in a subgroup with the highest dietary phytosterol intake (> 512 mg/d) than in a subgroup with the lowest intake (< 459 mg/d) (Sanclemente et al. 2012).

These findings indicate that at levels moderately low and achievable in normal Western diet, naturally occurring dietary phytosterols decrease the absorption of cholesterol in the gut and thereby the serum cholesterol levels as well. The decrease in serum cholesterol levels may reduce the risk of cardiovascular disease. Even modest increases in the dietary intake of phytosterols may have health-promoting effects.

Cancer preventive effects

Experimental and epidemiological studies indicate that dietary phytosterols may protect people from various types of cancer, such as colon, breast and prostate cancers (Awad and Fink 2000; Jones and AbuMweis 2009). Phytosterols may also inhibit lung, stomach and ovarian cancers (Woyengo et al. 2009). The exact mechanisms of action are not known, but the anticancer properties of phytosterols are possibly related to their ability to reduce the formation of carcinogens, to inhibit the growth and proliferation of cancer cells and to promote apoptosis (cell death) of cancer cells. Phytosterols were found to suppress the proliferation, migration and invasion and also to induce apoptosis of prostate cancer cells (Ifere et al. 2009; Lu and Zhang 2009). On the other hand, Normén et al. (2001) did not observe any relationship between the normal dietary intake of phytosterols and the risk of colon and rectal cancers in humans.

Inflammation preventive effects

Beneficial anti-inflammatory effects of dietary phytosterols have been suggested in animal and human studies, but their potential mechanisms of action are not fully understood (Othman and Moghadasian 2011). The protection against inflammatory-related conditions that cause cardiovascular disease may be based on the ability of phytosterols to modify the immune system. Phytosterols may also regulate the production of inflammatory mediators. These effects possibly result from the ability of phytosterols to control membrane properties, such as fluidity, sensitivity and signaling pathways. For example, sitosterol was shown to lower inflammation in mouse magrophages by down-regulating certain pro-inflammatory signaling pathways (Valerio and Awad 2011).

Other potential biological properties

Some effects of phytosterols on serum vitamin levels have been observed. Consumption of phytosterol-enriched foods may reduce the plasma concentration of ß-carotene (Jones and AbuMweis 2009; Gylling et al. 2010). There is also some evidence of a slight reduction in the concentrations of fat-soluble vitamins and an increase in folate and vitamin B12 concentrations in plasma (Derdemezis et al. 2010). Gylling et al. (2010) found that a high plant stanol ester intake (8.8 g/d) only lowered the serum ß-carotene levels but did not affect the concentrations of active vitamin A, vitamin D, α-carotene or γ-tocopherol in serum.

Derdemezis et al. (2010) reported the effects of phytosterols observed in cell cultures and animal and human studies. They may affect the endothelial function, platelet aggregation and formation of atherosclerotic plaque. In addition, anti-atherogenic, anti-ulcer, anti-fungal and anti-oxidative activities have been suggested (Berger et al. 2004). Stanol esters were found to reduce the level of oxidised LDL cholesterol in plasma in healthy humans (Homma et al. 2003). Phytosterols, such as Δ^5-avenasterol, that have an ethylidene group in their sidechain in C-24 were shown to exhibit antioxidant properties at high temperatures, but not at temperatures physiologically relevant to the human body (Kochhar 2000). The effects of phytosterols on oxidative stress have not been well established, and more animal and human studies are needed to clarify the antioxidant effects.

There has been much debate about the effect of plasma phytosterol concentration on the prevalence of cardiovascular disease. Phytosterols are virtually nonabsorbed in the human digestive system, and the levels of sterols in the blood are low compared to cholesterol. Approximately 56% of cholesterol is absorbed in the small intestine, whereas the absorption of sterols ranges from 0.51 to 1.89%, and that of stanols from 0.04 to 1.15% (Bosner et al. 1999; Ostlund et al. 2002). Several studies have suggested that the phytosterols present in the circulation either have no impact on the occurrence of cardiovascular disease or are associated with a reduced risk of cardiovascular disease (Fassbender et al. 2008; Silbernagel et al. 2009; Windler et al. 2009; Escurriol et al. 2010). A recent meta-analysis showed no associations between serum phytosterol concentrations and the risk of cardiovascular disease (Genser et al. 2012). Adverse statements have also been made (Weingärtner et al. 2008).

Steryl ferulates

Steryl ferulates combine the health-promoting effects of phytosterols and ferulic acid. Mammalian digestive steryl esterases cleave the ester bond between ferulic acid and sterol, liberating the cholesterol-lowering sterol moiety and antioxidative ferulic acid moiety (Moreau and Hicks 2004; Nyström et al. 2008; Mandak and Nyström 2012b). The health-enhancing potential of γ-oryzanol has been more widely studied than any other steryl ferulates (Lerma-García et al. 2009). Rice-derived γ-oryzanol is a commercial mixture of several steryl ferulates, which differs in composition from steryl ferulates found in other sources, such as wheat. According to Mandak and Nyström (2012b), the steryl ferulates of rice (containing a high proportion of 4,4'-dimethyl sterols) may not be as effectively hydrolysed in the digestive system as those of wheat and corn (containing desmethyl sterols), thus restricting their bioactivity in the gut. Furthermore, Lubinus et al. (2012) found that approximately 80% of ingested γ-oryzanol was found in intact nonhydrolysed form in the human feces. These results suggest that steryl ferulates from other sources than rice may e.g. lower cholesterol more efficiently.

Serum cholesterol-lowering effect

A small number of animal and human studies indicate that steryl ferulates have serum cholesterol-lowering properties, as do the free phytosterols and fatty acid esterified sterols in the diet. Since steryl ferulates are expected to be hydrolysed in the gut, their bioactivity should be similar to that of free phytosterols. A diet supplemented with phytostanyl ferulates (0.73%) decreased plasma total cholesterol levels by 15%, and a diet supplemented with steryl ferulate-rich corn fibre oil (10%) reduced cholesterol absorption by 24% and plasma total cholesterol levels by 29% in hamsters (Jain et al. 2008). Similarly, hamsters fed with diets containing 0.5 to 1% γ-oryzanol had a 28–44% reduction in the plasma total cholesterol levels and a significant reduction in aortic fatty streak formation (Rong et al. 1997). A decrease in plasma cholesterol levels was also observed in mildly hypercholesterolemic men after consuming rice bran oil containing low or high amounts (0.05 and 0.8 g/d, respectively) of γ-oryzanol (Berger et al. 2005). Consumption of high and low γ-oryzanol-containing oils both resulted in 6.3% lower total cholesterol and 10.5% lower LDL cholesterol levels. An equivalent amount of free sterols had a similar effect on cholesterol levels, indicating the deferulation of steryl ferulates in the digestive system. According to another study, however, free sterols lowered cholesterol absorption in hamsters more than phytosterols esterified with phenolic acids (Meijer et al. 2003). They also found soybean 4-desmethyl sterols more effective than rice bran 4,4'-dimethyl sterols in inhibiting cholesterol absorption and lowering serum cholesterol levels.

Antioxidant properties

Steryl ferulates are antioxidant compounds due to the radical scavenging ability of the ferulic acid moiety. Donation of a hydrogen atom from the phenolic *p*-hydroxyl group results in the formation of five resonance-stabilised steryl ferulate radicals (Kochhar 2000). Various steryl ferulates, including cycloartenyl ferulate and sitosteryl ferulate, had free radical-scavenging and antioxidative activities in the lipid membrane (Islam et al. 2009). Furthermore, cycloartenyl ferulate, ethyl ferulate and cycloartenol could inhibit the production of reactive oxygen species in living fibroblast cells. Steryl ferulates extracted from wheat and rye had antioxidant activity in bulk and emulsified lipid systems (Nyström et al. 2005). Rice bran steryl ferulates were shown to have antioxidative activity against cholesterol oxidation, thus reducing the formation of harmful cholesterol oxidation products, in *in vitro* cholesterol oxidation system (Xu et al. 2001). An antioxidant effect of rice bran steryl ferulates was also observed in a linoleic acid model (Xu and Godber 2001). Steryl ferulates may thus protect cells or cholesterol against oxidative stress, but there are no animal or human studies available (Ghatak and Panchal 2011).

Cancer preventive effects

Steryl ferulates have been suggested to have cancer-preventive properties. Cycloartenol ferulate originating from rice bran was shown to inhibit the growth of human colorectal adenocarcinoma by triggering apoptosis of colorectal cancer cells (Kong et al. 2009). Cycloartenol ferulate also inhibited tumor promotion in mice (Yasukawa et al. 1998). Various steryl ferulates separated from wheat, rye and corn bran oils were found to have inhibitory effects against tumor promotion in human lymphoblastoid cells (Iwatsuki et al. 2003).

Inflammation preventive effects

Anti-inflammatory properties of steryl ferulates have been demonstrated in a few studies. Rice bran steryl ferulates possessed inhibitory activity against inflammation in mice, showing higher activity than the corresponding free sterols (Akihisa et al. 2000). Rice bran extract and γ-oryzanol inhibited inflammation in mice and the effect of isolated individual steryl ferulates was dose-dependent (Yasukawa et al. 1998). In cell culture, cycloartenyl ferulate inhibited activation of the transcription factor involved in differentiation, proliferation, apoptosis and inflammation of cells and was suggested as a potential anti-inflammatory compound (Nagasaka et al. 2007). Islam et al. (2009) proposed that the

inflammation-preventive activity of steryl ferulates results from the ability of steryl ferulates to scavenge reactive oxygen species in living cells, thus protecting the cells from free radicals.

Other potential biological properties

Other possible biological effects of steryl ferulates include the prevention of liver injury induced by ethanol ingestion in mice (Chotimarkorn and Ushio 2008). An antimicrobial effect has also been suggested (Seitz 1989). Steryl ferulates reduced gastric ulcers and inhibited gastric acid secretion in animal models and may also have anti-diabetic, anti-aging and other protective effects owing to their antioxidative properties (Ghatak and Panchal 2011).

2.1.3 Intake and natural sources

Phytosterols

The estimates for daily phytosterol intake in European diets vary from 150 to 340 mg (Table 2), depending on population and gender. The lowest intakes were observed in British and Dutch diets (Morton et al. 1995; Schothorst and Jekel 1999), but more recently published studies of the same populations have given considerably higher values (Normén et al. 2001; Andersson et al. 2004; Klingberg et al. 2007). The intake of phytosterols was higher within men than within women (Table 2). The daily amount of phytosterols ranged from 212 to 311 mg for women and from 252 to 338 mg for men (when separately reported). Educational level and region, but not age, were shown to affect the daily intake in Finland (Valsta et al. 2004). The main dietary phytosterol is sitosterol, which comprised 57 to 66% of the total daily intake of phytosterols (Normén et al. 2001; Valsta et al. 2004; Klingberg et al. 2007; Wang et al. 2012). The relative proportions of campesterol, stanols and stigmasterol of the total sterol intake were 15–23, 5–12 and 3–11%, respectively.

Table 2. The mean daily total intake of phytosterols (mg/d) and the contribution of cereal products (%) to the intake.

Population	Year	Mean intake of phytosterols[a]	Cereal products (%)	Reference
British	1991	185.7	33.3	Morton et al. 1995
Dutch	1994	146		Schothorst and Jekel 1999
Dutch	1986-1992	285.0 262.9 (women) 307.3 (men)	38	Normén et al. 2001
Finnish	1997	237 (women) 305 (men)	42	Valsta et al. 2004
British	1993-1997	303 (women) 310 (men)		Andersson et al. 2004
Spanish	2000	275.5	29.7	Jiménez-Escrig et al. 2006
British	1993-1997	295.8 292.6 (women) 299.8 (men)	18.6	Klingberg et al. 2007
Swedish	1992-2005	212 (women) 252 (men)		Klingberg et al. 2008
Spanish	1992-1996	250.4 (women) 337.9 (men)		Escurriol et al. 2009b
Chinese	2005-2009	317 311 (women) 330 (men)	34.7	Wang et al. 2012

[a] Differences in the sterol analysis methods, number of sterols analysed and dietary assessment methods.

The major dietary sources of phytosterols include the following: 1) vegetable oils, margarines and vegetable-fat spreads, 2) bread and other cereal products and 3) fruits and vegetables. Other sources of phytosterols include nuts, seeds and almonds. The cereals are only moderate in their sterol content when compared to vegetable oils, but they provide a significant amount of sterols to the diet due to the high amount typically consumed. In Finnish, Dutch and Chinese diets, the cereal products were the most important natural source of phytosterols, accounting for up to 42% of the total daily intake (Table 2). The high consumption of rye and wholegrain products contributed to the high phytosterol intake in Finland and the Netherlands. Lower values were reported in British and Spanish diets; 19 to 33% of the daily intake was obtained from cereal products. The main sources of phytosterols in Spain, for example, were vegetable oils. The contribution of vegetable oils and margarines to the average daily phytosterol intake varied from 17 to 39% in the Western diet, and vegetables and fruits provided 11–18% and 10–12%, respectively, of the total intake (Normén et al. 2001; Valsta et al. 2004; Jiménez-Escrig et al. 2006; Klingberg et al. 2007). Approximately 30% of total phytosterols obtained in the Chinese diet was from vegetables, fruits and legumes, 27% was from vegetable oils and 7% was from nuts (Wang et al. 2012).

Due to their health-promoting effects, phytosterols are added to functional food products (e.g. spreads, yoghurts, cheese and mayonnaise). The excessive consumption of sterol-enriched products may lead to high daily doses of phytosterols, which are above the current recommendations. The Irish consumers of phytosterol-enriched products obtained an average of 2.45 g/d phytosterols, but some of the consumers had exceptionally high intakes, up to 9.84 g/d (Hearty et al. 2009). It has been estimated that the simultaneous usage of multiple sterol-enriched foods can result in a daily supply of 13 g phytosterols (Kuhlmann et al. 2005). The long-term consequences of such high intakes are not yet known. At the same time, consumers are increasingly calling for naturalness and natural food (Mellentin 2012).

Being one of the most important sources of phytosterols, cereals play a significant role in increasing the intake of natural phytosterols. Cereal products also contain a great number of other health-protective compounds, such as dietary fibre, minerals, vitamins and phenolic compounds (Slavin 2004; Fardet 2010). Wholegrain products are particularly good sources of phytosterols, since sterols are suggested to concentrate in the germ, bran and aleurone (Singh et al. 2001; Nyström et al. 2007).

The bioaccessibility of sterols in the gut may differ depending on the food matrix. The bioactivity of phytosterols in functional foods has been better established when phytosterols were introduced in matrices such as vegetable oil-based spreads and milk products compared to cereal products (AbuMweis et al. 2008; EFSA 2009). The bioaccessibility of natural sterols from cereal matrices is not known, but phytosterols obtained from sources such as vegetable oils may be more available to express bioactivity in the gastrointestinal tract. Therefore, the bioaccessibility of phytosterols from cereal products needs to be clarified.

Steryl ferulates

The total daily phytosterol intakes have been estimated in several studies (Table 2). However, assessments on the intakes of steryl ferulate conjugates have not been published. Steryl ferulates are exclusively obtained from certain cereals grains and seeds. Rice steryl ferulates, i.e. γ-oryzanol, have been widely studied (Lerma-García et al. 2009). Other sources include corn, wheat, rye, and more uncommon cereal types, such as triticale, wild rice, Job's tears and teosinte (Seitz 1989; Moreau et al. 1998; Moreau et al. 2001; Hakala et al. 2002; Werner et al. 2002). Low contents have also been reported in barley (Moreau et al. 1998).

2.2 Phytosterols and steryl ferulates in wheat

Cereals are an important source of natural phytosterols and their ferulic acid esters, along with other bioactive phytochemicals such as dietary fibre, tocols and folate (Ward et al. 2008). Wheat is one of the major cereal grains consumed in Europe and throughout the world and thus contributes to the intake of phytosterol compounds, especially in the Western diet.

2.2.1 Occurrence in wheat

Phytosterols

A majority of the sterols found in wheat are desmethyl sterols; 4-Monomethyl and 4'4-dimethyl sterols serve as precursors of desmethyl sterols and are found in low amounts. The main phytosterol species in wheat is sitosterol, followed by campesterol, the corresponding saturated stanol forms and stigmasterol (Figure 4). Other species include brassicasterol, Δ^5-avenasterol, cycloartenol, Δ^7-stigmastenol, Δ^7-avenasterol and 24-methylenecycloartanol (Piironen et al. 2002). Several other minor sterol species have been identified in wheat, namely stigmastadienol, gramisterol, α-amyrin and citrostadienol (Nyström et al. 2007). In addition, cholesterol and ergosterol were found in spelt and winter wheat (Rozenberg et al. 2003; Ruibal-Mendieta et al. 2004). The ergosterol, however, was probably present because of microbial contamination, since ergosterol occurs in yeast and other fungi (Moreau et al. 2002). In wheat, phytosterols exist as free sterols, fatty acid or hydroxycinnamic acid esterified conjugates, glycosides or acylated glycosides (Ruibal-Mendieta et al. 2004; Nyström et al. 2007).

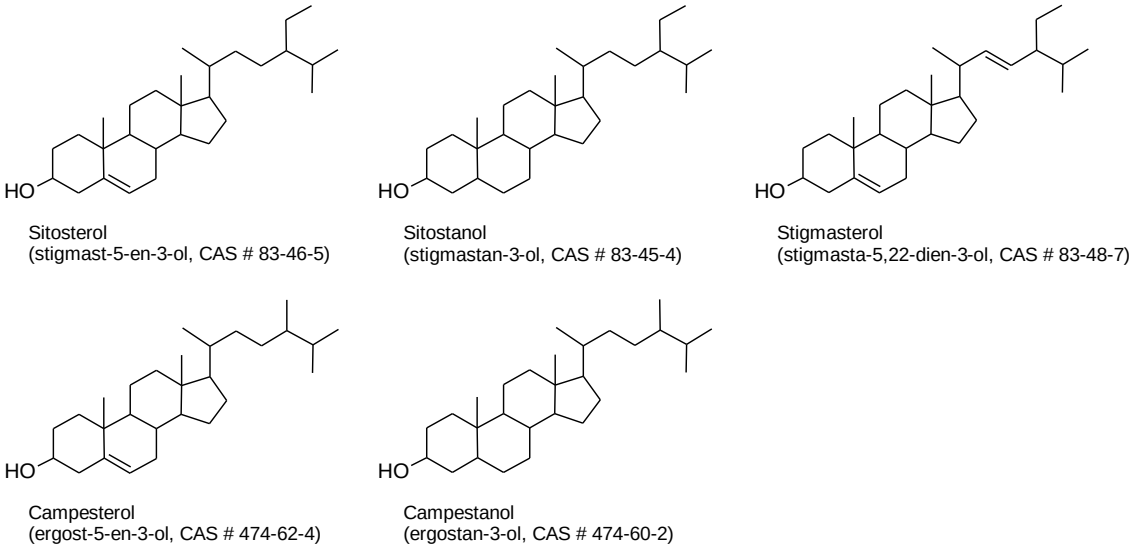

Figure 4. Chemical structures of the main phytosterols present in wheat.

Steryl ferulates

Four main steryl ferulate species have been identified in wheat (Seitz 1989; Hakala et al. 2002). The structures of these compounds are presented in Figure 5. Campestanyl ferulate and sitostanyl ferulate are the most abundant compounds, whereas the corresponding sterol species are present in lower quantities. The reason for the preferential esterification of stanols compared to sterols is not known. Recently, coumaric acid esters of phytosterols and stanols, namely trans-campestanyl and sitostanyl coumarates, were identified in wheat as minor components (Esche et al. 2012). In addition, small amounts of 24-methylene cycloartanyl, Δ^7-campesteryl and Δ^7-sitosteryl ferulates were detected. Only the ferulate conjugates were observed in spelt (Esche et al. 2012). The trans-isomers of steryl and stanyl ferulates dominated over the cis-isomers.

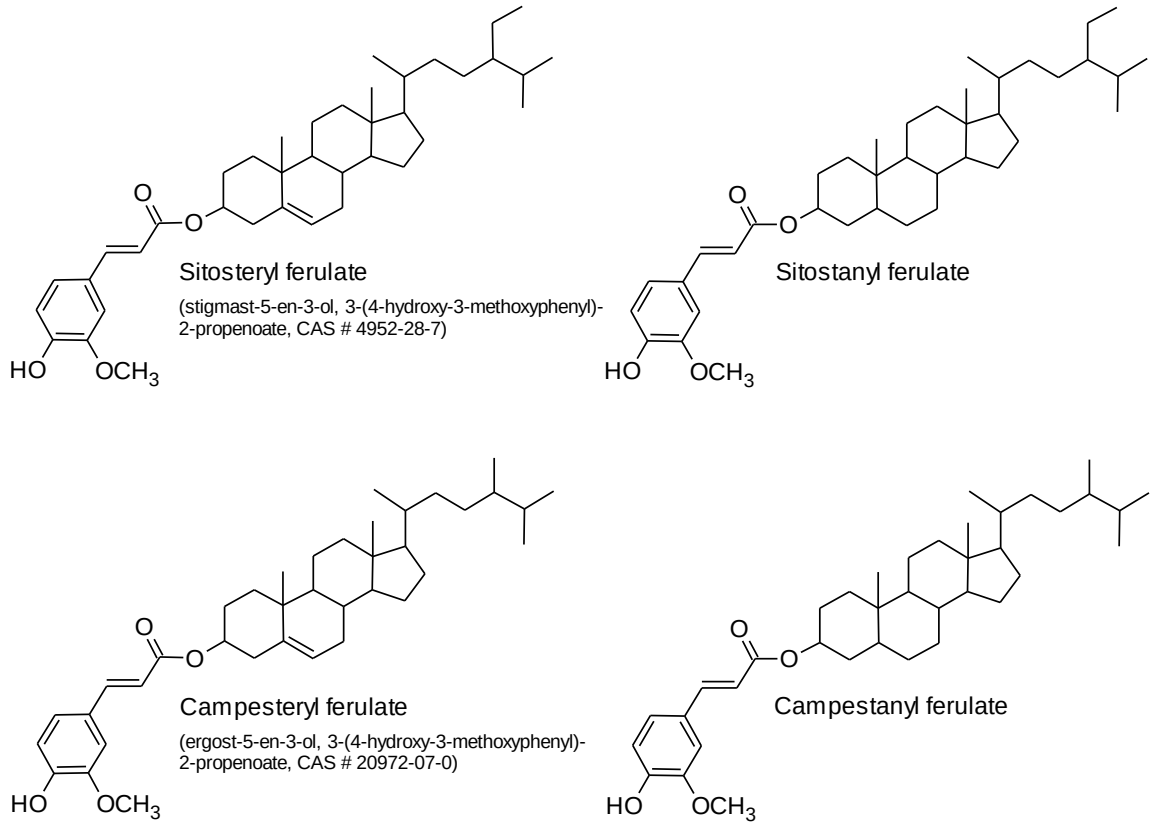

Figure 5. Chemical structures of the main steryl ferulates present in wheat.

2.2.2 Contents in wholegrain wheat

Phytosterols

In common bread wheat, phytosterol contents ranging from approximately 600 to 800 µg/g have been generally reported, either on a dry matter or a wet basis (Table 3). These studies have included various wheat samples with single, mixed or unspecified varieties and samples delivered from the experimental fields, mills or local grocery stores. The comparison of the values is also difficult due to differences in the analytical methods and the number of compounds analysed. In an analysis of cereal samples, both acid and base hydrolyses are required to liberate the glycosidic and esterified conjugates of phytosterols (Toivo et al. 2001). Thus, exclusion of acid hydrolysis in sample preparation underestimates the total content of sterols. In one study, for example, free phytosterols (225 and 214 µg/g DM), intact steryl fatty acid esters (370 and 558 µg/g DM) and intact phenolic acid esters (124 and 95 µg/g DM) were simultaneously analysed in wheat and spelt, respectively, but the glycosidic conjugates were excluded (Esche et al. 2012). In another study, free and esterified sterol fraction and steryl glycoside and acylated steryl glycoside fraction were determined separately, including all conjugates (Ruibal-Mendieta et al. 2004). When determination of intact conjugates is not necessary, a sample procedure with acid and base hydrolysis will give the amount of total sterols. The differences in the levels of phytosterols may also result from differences in environmental conditions and cultivars analysed.

One study compared the phytosterol contents of four different wheat types; bread wheat, durum wheat, spelt and emmer wheat (Iafelice et al. 2009). Durum wheat, spelt and emmer wheat showed slightly higher levels of phytosterols than common bread wheat. In general, however, the total sterol content of spelt was similar to that of bread wheat, varying approximately from 600 to 800 µg/g (Ruibal-Mendieta et al. 2004; Ryan et al. 2007; Iafelice et al. 2009).

Sitosterol was the most abundant sterol species in all wheat types, comprising approximately 60% of the total content of phytosterols in bread wheat (Table 3). One of the studies suggested a relative content as high as 83% for sitosterol, but the authors only analysed three main sterols, thus overestimating the proportion of sitosterol (Chen et al. 2009). Compared to bread wheat, emmer and durum wheat had slightly lower relative contents of sitosterol, 40 to 50%, whereas in spelt and bread wheat, content was equal (Table 3). These values, however, were obtained from one to three studies only. Campesterol accounted for approximately 15 to 20% and stanols (i.e., sitostanol and

campestanol) made up 15 to 20% of the total content. As an exception, durum and emmer wheat showed slightly higher relative content of stanols (28–34%) than other wheat types. One study reported exceptionally low stanol contents in winter wheat and spelt (Ruibal-Mendieta et al. 2004). The relative content of stigmasterol was up to 5% in all wheat types. Other minor sterols have not been reported in most of the studies, but the total content for sterols other than sitosterol, campesterol and stanols was suggested to be 10% in wholegrain wheat (Nyström et al. 2007).

Steryl ferulates

In wholegrain wheat, 6 to 7% of the total phytosterols were esterified with ferulic acid (Hakala et al. 2002; Nyström et al. 2007). The steryl ferulates of wheat have only been investigated in a few studies (Table 4). The total content of steryl ferulates were shown to vary approximately from 50 to 120 µg/g in bread wheat. For durum and spelt wheat, similar values have been reported. One study reported the amount of sterols as steryl ferulates instead of exact steryl ferulate content (Nyström et al. 2007). This value (52 µg/g DM) can be converted to the amount of steryl ferulates, the total content being 74 µg/g DM.

Most of the studies reported the content of the predominant compound campestanyl ferulate as a sum with coeluting sitosteryl ferulate, and one of the studies reported this value separately (Table 4). In total, campestanyl and sitosteryl ferulates account for 50 to 60% of all steryl ferulates. Of this amount, approximately 77–85% is covered by campestanyl ferulate according to mass spectral analyses (Seitz 1989; Hakala et al. 2002). The relative content of sitostanyl ferulate is 30–35% and that of campesteryl ferulate is 10–20%. One study suggested that wheat also contains steryl coumarates (2%) and 24-methylene cycloartanyl ferulate, Δ^7-campesteryl and Δ^7-sitosteryl ferulates (4%) as minor compounds (Esche et al. 2012). Spelt contained Δ^7-campesteryl and Δ^7-sitosteryl ferulates (3%).

Table 3. Phytosterol composition of wholegrain wheat, presented as µg/g of either fresh weight (FW) or dry matter (DM). The relative contents (%) of phytosterols are given in parentheses (continues).

Sample type	N[a]	Sitosterol	Campesterol	Sitostanol	Campestanol	Total stanols	Stigmasterol	Total phytosterols		Reference
Bread wheat										
Whole wheat	1	400 (58%)	270 (39%)	n.r. [b]	n.r.	n.r.	0	690	FW	Weihrauch and Gardner 1978
Wheat grain	2	n.r.	n.r.	n.r.	n.r.	n.r.	n.r.	592–638	FW	Hakala et al. 2002
Whole wheat flour	1	440 (63%)	140 (20%)	110 (16%)	0	110 (16%)	17 (2%)	700	FW	Normén et al. 2002
Wholegrain wheat (grains and flour)	5	360–486 (51–59%)	108–150 (16–18%)	83–112 (12–16%)	59–73 (8–11%)	151–171 (21–24%)	15–22 (2–3%)	665–830	FW	Piironen et al. 2002
Winter wheat wholemeal	5	431–466 (67–73%)	140–174 (22–27%)	6–16 (1–3%)	7–17 (1–3%)	14–31 (2–5%)	3 (0–1%)	622–655	n.r.	Ruibal-Mendieta et al. 2004
Wheat, whole grain	2	413 (53%)	125 (16%)	n.r.	n.r.	168 (22%)	n.r.	783	DM	Nyström et al. 2007
Wholegrain wheat	23	264–435	68–143	42–130	24–79	n.r.	13–28	494–796	DM	Alignan et al. 2009
Wholegrain wheat	12	150–283 (73–83%)	35–63 (14–24%)	n.r.	n.r.	n.r.	5–10 (2–5%)	196–355	FW	Chen et al. 2009
Wheat	5	348–402 (58–60%)	86–138 (14–20%)	57–88 (8–14%)	30–47 (5–8%)	94–132 (14–21%)	7–10 (1%)	600–677	DM	Iafelice et al. 2009
Wheat kernels	164[c]	346 (57%)	105 (17%)	56 (9%)	37 (6%)	93 (15%)	22 (4%)	608	FW	Plumb et al. 2011

Table 3. (Continued) Phytosterol composition of wholegrain wheat, presented as µg/g of either fresh weight (FW) or dry matter (DM). The relative contents (%) of phytosterols are given in parentheses.

Sample type	N	Sitosterol	Campesterol	Sitostanol	Campestanol	Total stanols	Stigmasterol	Total phytosterols		Reference
Other wheat types										
Spelt	16	385–567 (69–73%)	122–191 (19–24%)	6–20 (1–3%)	7–20 (1–2%)	14–39 (3–5%)	2–4 (0–1%)	544–807	n.r.	Ruibal-Mendieta et al. 2004
Spelt	1	533 (77%)	151 (22%)	n.r.	n.r.	n.r.	4 (1%)	688	n.r.	Ryan et al. 2007
Spelt	12	325–476 (52–60%)	78–137 (12–17%)	77–128 (11–20%)	47–68 (6–10%)	124–189 (18–29%)	9–17 (1–3%)	628–819	DM	Iafelice et al. 2009
Emmer	9	314–435 (42–51%)	105–165 (15–17%)	122–165 (17–21%)	81–144 (11–15%)	203–309 (28–34%)	13–17 (2 %)	683–957	DM	Iafelice et al. 2009
Durum	5	326–411 (44-50%)	125–149 (17–18%)	118–135 (15–18%)	96–117 (13–16%)	214–252 (28–34%)	14–16 (2%)	737–840	DM	Iafelice et al. 2009

[a] Number of samples
[b] Not reported
[c] Number of datapoints from the EuroFIR BASIS database.

Table 4. Steryl ferulate composition of wholegrain wheat, presented as µg/g of either fresh weight (FW) or dry matter (DM). The relative contents (%) of steryl ferulates are given in parentheses.

Sample type	N[a]	Campestanyl ferulate	Sitosteryl ferulate	Total campestanyl and sitosteryl ferulates	Sitostanyl ferulate	Campesteryl ferulate	Total steryl ferulates		Reference
Bread wheat									
Winter wheat	6	n.r.[b]	n.r.	35–53 (54–60%)	19–32 (30–34%)	6–9 (10–13%)	62–94	n.r.	Seitz 1989
Spring wheat	1	n.r.	n.r.	66 (54%)	44 (36%)	12 (10%)	123	n.r.	Seitz 1989
Wheat (w/o hull)	1	n.r.	n.r.	n.r.	n.r.	n.r.	53	FW	Moreau et al. 1998
Wheat grain	2	n.r.	n.r.	32–33 (52%)	18–19 (29–31%)	11–12 (18–19%)	62–63	FW	Hakala et al. 2002
Wheat, whole grain	2	n.r.	n.r.	26 (50%)	17 (33%)	9 (17%)	52[c]	DM	Nyström et al. 2007
Wheat kernels	1	56 (45%)	6 (5%)	62 (50%)	42 (34%)	13 (10%)	124	DM	Esche et al. 2012
Other wheat types									
Durum wheat	1	n.r.	n.r.	44 (64%)	20 (29%)	5 (7%)	68	n.r.	Seitz 1989
Spelt kernels	1	40 (44%)	4 (5%)	45 (48%)	36 (39%)	8 (9%)	92	DM	Esche et al. 2012

[a] Number of samples
[b] Not reported
[c] Content of sterols as steryl ferulates

2.2.3 Contents in wheat fractions

The conventional milling process separates the white endosperm-rich flour from the outer layers of the grain. The bran is a by-product, which is removed and used as feed, although it is a rich source of fibre, phytochemicals and minerals. Apart from the conventional wheat milling, alternative dry processes can be used to produce various wheat grain and bran fractions with different compositions (Dexter and Wood 1996; Hemery et al. 2007; Anson et al. 2012; Brouns et al. 2012).

Phytosterols

The phytosterol contents of the wheat dry fractionation products in previous studies have varied considerably, from 300 to 4900 µg/g (Table 5). Wheat germ and bran are especially rich in phytosterols, containing 2400–4900 and 1200–2000 µg/g phytosterols, respectively. The aleurone fraction separated from bran also contains high levels of sterols. The poorest sources were refined wheat flours with low ash content, which contain approximately half of the amount of phytosterols compared to wholegrain wheat (Table 3). Total phytosterol content of flours increases with increasing ash content. The bran-rich fractions with varying ash and fibre contents obtained in various stages of the conventional milling process also had significant differences in phytosterol contents; the higher the fibre and ash contents, the higher the total phytosterol content (Nyström et al. 2007). In wheat germ oil, very high sterol contents have been reported.

The sterol composition varied in wheat fractions (Table 5). The main compound sitosterol accounts for 38 to 68% of total sterols depending on the wheat fraction. The sterol profile of wheat germ and flour fractions is characterised by a high content of sitosterol, whereas in bran fractions, the relative content of sitosterol is lower. The relative content of campesterol (12–27%) is higher in the germ than in other wheat fractions. Germ is poor in stanols, since only 2–7% of the total content is covered by sitostanol and campestanol. Stanols comprise up to 37% of total sterols in bran and up to 16% in flour fractions. The relative content of stigmasterol varies from 0–4% depending on the wheat fraction.

Available knowledge shows that the total content and composition of phytosterols vary considerably among various wheat fractions: endosperm, bran and germ. However, not much is known of the variation in more specific fractions of wheat, e.g. in bran that is further fractionated to yield various subfractions. Such bran fractions can be produced in conventional and novel dry processes. As an example, phytosterol-rich aleurone can be

separated from the bran (Buri et al. 2004a). More research is needed in this area to identify potential wheat fractions with enriched phytochemical contents.

Steryl ferulates

Steryl ferulates accounted for 13 to 17% of wheat bran phytosterols, which is a considerably higher value than in wholegrain flour (Hakala et al. 2002; Nyström et al. 2007). Table 6 shows that only a few studies are available about steryl ferulates in wheat fractions. Wheat bran has been reported to contain from 300 to 600 µg/g steryl ferulates. The contents in aleurone fractions separated from bran were slightly lower than in the total bran fraction. In refined flour with low ash content, only traces of steryl ferulates were found, whereas the enriched flour with high ash content had relatively high levels of steryl ferulates, approximately 200 µg/g FW. Such a value is 2- to 3-fold compared to the steryl ferulate content of wholegrain wheat flour (Table 4). Wheat germ was poor in steryl ferulates, probably due to the accumulation of ferulic acid in the bran (Barron et al. 2007). One study reported the contents of sterols as steryl ferulates, and when converted to steryl ferulates, the total contents in bran fractions were 424 to 438 µg/g DM, 24 µg/g DM in the germ and 17–81 µg/g DM in wheat milling fractions (Nyström et al. 2007).

In wheat flour, bran and germ, approximately half of the total steryl ferulates was covered by campestanyl ferulate and coeluting sitosteryl ferulate (Table 6). In two previous studies these two compounds were quantified individually; the relative content of campestanyl was found to be 42% in bran (Collins et al. 2002; Jiang and Wang 2005). This is in line with the findings of Hakala et al (2002) and Seitz et al. (1989), since their mass spectrometric analyses indicated that campestanyl ferulate accounted for 77 to 85% of the mixture. Sitostanyl ferulate comprised 28 to 33% of the total steryl ferulates in the bran, germ and milling fractions and slightly more (36–37%) in the aleurone fractions (Table 6). The relative content of campesteryl ferulate ranged from 14 to 20%, depending on the fraction.

Table 5. The phytosterol composition of various wheat fractions, presented as µg/g of either fresh weight (FW) or dry matter (DM). The relative contents (%) of phytosterols are given in parentheses (continues).

Sample type	N[a]	Sitosterol	Campesterol	Sitostanol	Campestanol	Total stanols	Stigmasterol	Total phytosterols		Reference
Flour fractions										
Wheat flour	1	n.r.[b]	n.r.	n.r.	n.r.	n.r.	n.r.	600	FW	Weihrauch and Gardner 1978
Wheat flour	1	190 (68%)	47 (17%)	0	0	0	44 (16%)	280	FW	Normén et al. 2002
Wheat flour, 0.6% ash	2	n.r.	n.r.	n.r.	n.r.	n.r.	n.r.	330–349	FW	Hakala et al. 2002
Wheat flour, 0.6–0.7% ash	6	228–321 (61–67%)	63–85 (16–18%)	33-49 (8–10%)	20–31 (5–7%)	58–80 (14–16%)	5–7 (0–1%)	373–507	FW	Piironen et al. 2002
Wheat flour, 1.2–1.4% ash	3	368–452 (61–64%)	111-153 (18-22%)	48–65 (8–9%)	18-40 (4-7%)	88–93 (13–14%)	8–9 (1%)	607–704	FW	Piironen et al. 2002
Wheat enriched flour, 4.5% ash	2	n.r.	n.r.	n.r.	n.r.	n.r.	n.r.	1679–1823	FW	Hakala et al. 2002
Bran fractions										
Wheat bran	2	n.r.	n.r.	n.r.	n.r.	n.r.	n.r.	890–1540	FW	Weihrauch and Gardner 1978
Wheat bran	4	n.r.	n.r.	n.r.	n.r.	n.r.	n.r.	1479–1668	FW	Hakala et al. 2002
Wheat bran coarse	1	820 (42%)	260 (13%)	440 (22%)	380 (19%)	820 (42%)	70 (4%)	1970	FW	Normén et al. 2002
Wheat bran	1	990 (50%)	360 (18%)	310 (16%)	270 (14%)	580 (29%)	72 (4%)	2000	FW	Normén et al. 2002

Table 5. (Continued) The phytosterol composition of various wheat fractions, presented as µg/g of either fresh weight (FW) or dry matter (DM). The relative contents (%) of phytosterols are given in parentheses (continues).

Sample type	N	Sitosterol	Campesterol	Sitostanol	Campestanol	Total stanols	Stigmasterol	Total phytosterols		Reference
Wheat bran	5	704–938 (42–52%)	243–354 (13–20%)	207–390 (11–20%)	165–338 (9–17%)	372–728 (21–37%)	12–74 (1–4%)	1678–1951	FW	Piironen et al. 2002
Wheat bran	1	n.r.	n.r.	n.r.	n.r.	n.r.	n.r.	1200	FW	Jiang and Wang 2005
Fine wheat bran	2	907 (44%)	310 (15%)	n.r.	n.r.	569 (27%)	n.r.	2075	DM	Nyström et al. 2007
Coarse wheat bran	2	700 (39%)	224 (13%)	n.r.	n.r.	628 (35%)	n.r.	1789	DM	Nyström et al. 2007
Wheat bran	2	662-716 (38–41%)	232–258 (12–16%)	237–374 (15–20%)	259–333 (16–18%)	496–707 (31–37%)	62–74 (4%)	1600–1900	DM	Kamal-Eldin et al. 2009
Wheat bran	49[c]	858 (48%)	292 (16%)	288 (16%)	241 (14%)	529 (30%)	53 (3%)	1783	FW	Plumb et al. 2011
Total lipid extract of wheat bran	1	4530 (26%)	3660 (21%)	2450 (14%)	1740 (10%)	4190 (24%)	270 (2%)	17670[d]		Jiang and Wang 2005
Germ										
Wheat germ	1	2300 (67%)	940 (27%)	69 (2%)	99 (3%)	168 (5%)	32 (1%)	3440	FW	Normén et al. 2002
Wheat germ	1	2537 (62%)	1034 (25%)	68 (2%)	54 (1%)	122 (3%)	19 (0%)	4114	FW	Piironen et al. 2002
Wheat germ	1	n.r.	n.r.	n.r.	n.r.	n.r.	n.r.	2400	FW	Jiang and Wang 2005
Wheat germ	2	2954 (60%)	1167 (24%)	n.r.	n.r.	109 (2%)	n.r.	4923	DM	Nyström et al. 2007

Table 5. (Continued) The phytosterol composition of various wheat fractions, presented as µg/g of either fresh weight (FW) or dry matter (DM). The relative contents (%) of phytosterols are given in parentheses.

Sample type	N	Sitosterol	Campesterol	Sitostanol	Campestanol	Total stanols	Stigmasterol	Total phytosterols		Reference
Wheat germ	25[c]	2374 (65%)	829 (23%)	84 (2%)	62 (2%)	146 (4%)	29 (1%)	3640	FW	Plumb et al. 2011
Wheat germ oil	1	13200 (67%)	4330 (22%)	n.r.	n.r.	n.r.	tr[e]	19700		Weihrauch and Gardner 1978
Wheat germ oil refined	1	3700 (67%)	1220 (22%)	n.r.	n.r.	n.r.	tr	5530		Weihrauch and Gardner 1978
Total lipid extract of wheat germ	1	11230 (53%)	4700 (22%)	830 (4%)	670 (3%)	1500 (7%)	220 (1%)	21280[d]		Jiang and Wang 2005
Wheat germ oil	1	5040 (63%)	2160 (27%)	n.r.	n.r.	n.r.	64 (1%)	8000		Hassanein and Abedel-Razek 2009
Other fractions										
Aleurone standard	1	979 (45%)	254 (12%)	271 (12%)	422 (19%)	693 (32%)	47 (2%)	2193	n.r.	Buri et al. 2004a
Total lipid extract of durum wheat[f]	1	4710 (31%)	2180 (14%)	2100 (14%)	2900 (19%)	5000 (33%)	350 (2%)	15070[d]		Jiang and Wang 2005
Durum wheat[f]	1	n.r.	n.r.	n.r.	n.r.	n.r.	n.r.	1800	FW	Jiang and Wang 2005
Wheat milling fractions[g]	7	494–1021 (56–60%)	152–355 (17–20%)	n.r.	n.r.	95–204 (10–14%)	n.r.	817–1797	DM	Nyström et al. 2007

[a] Number of samples
[b] Not reported
[c] Number of datapoints from the EuroFIR BASIS database
[d] Given as µg sterols/g lipids
[e] Traces
[f] Mixture of germ and bran
[g] Fractions taken after the 6th, 9th, 10th and 11th smooth roll, after the 3rd divider and after the bran finisher, and dark wheat flour

Table 6. Steryl ferulate composition of various wheat fractions, presented as µg/g of either fresh weight (FW) or dry matter (DM). The relative contents (%) of steryl ferulates are given in parentheses (continues).

Sample type	N[a]	Campestanyl ferulate	Sitosteryl ferulate	Total campestanyl and sitosteryl ferulates	Sitostanyl ferulate	Campesteryl ferulate	Total steryl ferulates		Reference
Flour fractions									
Wheat flour, 0.6% ash	2	n.r.[b]	n.r.	tr[c]	tr	tr	< 5	FW	Hakala et al. 2002
Wheat enriched flour, 4.5% ash	2	n.r.	n.r.	98–110 (51%)	58–62 (29–30%)	38–44 (20%)	194–216	FW	Hakala et al. 2002
Bran fractions									
Wheat bran	1	247 (42%)	54 (9%)	301 (52%)	163 (28%)	120 (21%)	584	DM	Collins et al. 2002
Wheat bran	4	n.r.	n.r.	148–200 (50–51%)	91–114 (29–31%)	57–79 (19–21%)	297–390	FW	Hakala et al. 2002
Fine wheat bran	2	n.r.	n.r.	157 (53%)	98 (33%)	44 (15%)	298[d]	DM	Nyström et al. 2007
Coarse wheat bran	2	n.r.	n.r.	161 (52%)	103 (33%)	44 (14%)	308[d]	DM	Nyström et al. 2007
Total lipid extract of wheat bran	1	960 (47%)	140 (7%)	1100 (54%)	790 (39%)	160 (8%)	2050[e]		Jiang and Wang 2005
Germ									
Wheat germ	2	n.r.	n.r.	9 (53%)	5 (29%)	3 (18%)	17[d]	DM	Nyström et al. 2007

Table 6. (Continued) Steryl ferulate composition of various wheat fractions, presented as µg/g of either fresh weight (FW) or dry matter (DM). The relative contents (%) of phytosterols are given in parentheses.

Sample type	N	Campestanyl ferulate	Sitosteryl ferulate	Total campestanyl and sitosteryl ferulates	Sitostanyl ferulate	Campesteryl ferulate	Total steryl ferulates		Reference
Other fractions									
Aleurone standard	1	n.r.	n.r.	120 (46%)	96 (37%)	36 (14%)	260	n.r.	Buri et al. 2004a
Aleurone highly pure	1	n.r.	n.r.	128 (50%)	93 (36%)	35 (14%)	258	n.r.	Buri et al. 2004a
Wheat milling fractions[f]	7	n.r.	n.r.	6–30 (45–61%)	4–18 (27–33%)	2–9 (11–23%)	12–57[d]	DM	Nyström et al. 2007
Total lipid extract of durum wheat[g]	1	970 (66%)	50 (3%)	1020 (69%)	400 (27%)	30 (2%)	1470[e]		Jiang and Wang 2005

[a] Number of samples
[b] Not reported
[c] Traces
[d] Content of sterols as steryl ferulates
[e] Given as µg free sterols/g lipids
[f] Fractions taken after the 6th, 9th, 10th and 11th smooth roll, after the 3rd divider and after the bran finisher, and dark wheat flour
[g] Mixture of germ and bran

2.3 Variation of phytosterols and steryl ferulates in wheat

Natural variation creates a basis for enrichment of phytochemicals in foods. During the grain's development, wheat is exposed to various internal and external factors, which may affect its forthcoming properties and characteristics. Genotype and growing environment, as well as processing, may have an impact on the phytochemical composition of the resulting cereal products (Figure 6).

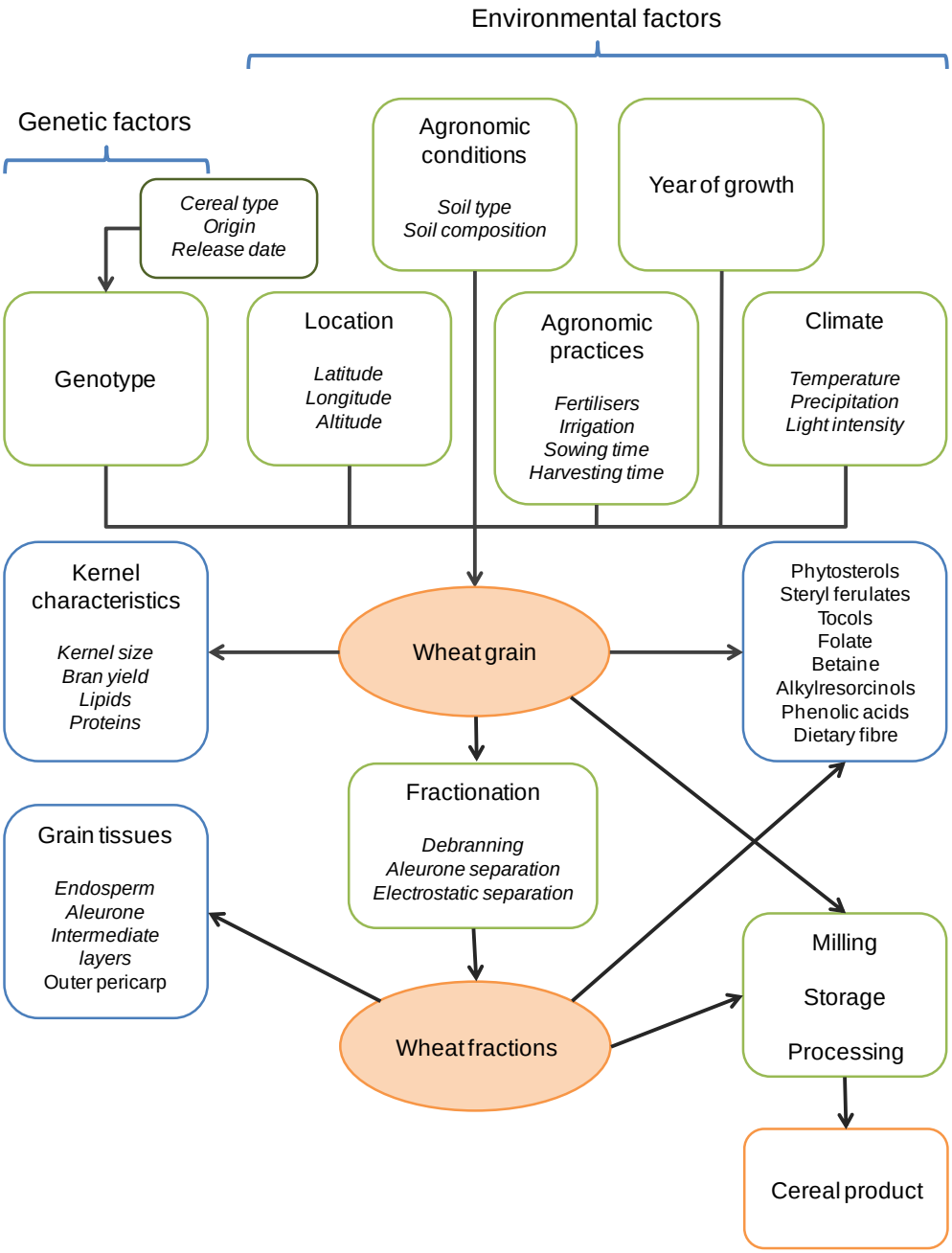

Figure 6. Possible genetic and environmental factors (with examples) affecting wheat grains during development and processing factors having impact on resulting cereal products (green boxes). Examples of the phytochemicals and kernel characteristics determined during the HEALTHGRAIN project are given (blue boxes).

2.3.1 Genetic variation

When wheat genotypes are grown in the same fields at a single location during one growing season, the variation occurring in their phytochemical composition is likely to arise from genetic factors. Genotypes include varieties or cultivars, breeding lines, traditional landraces, germplasms and synthetic populations. Over 25 000 individual bread wheat varieties have been developed worldwide (Shewry 2009b). Modern cultivars have been selected based on high yields and favorable end-use qualities.

Phytosterols

Only five studies have investigated the genetic variation of phytosterols in bread wheat, mainly within European cultivars (Table 7). In all except one, a limited number of genotypes were studied. Within these studies, the widest variation in sterol contents was observed among 23 wheat cultivars and synthetic populations grown under organic conditions in France, which was possibly due to the greater number of genotypes included in the trial (Alignan et al. 2009). Only modest variation was found in the sterol contents of five winter wheat cultivars grown in Belgium (Ruibal-Mendieta et al. 2004). Similarly, the total phytosterol contents of two Finnish cultivars grown in Finland in 1997, five Italian cultivars grown in Italy in 2002 and three cultivars from the United States of America (USA) grown in 2005 did not exhibit much variation (Piironen et al. 2002; Chen et al. 2009; Iafelice et al. 2009). However, genetic variation was reported to be statistically significant in two of these studies (Chen et al. 2009; Iafelice et al. 2009). Wheat types other than bread wheat, namely, spelt, durum wheat and ancient emmer wheat, exhibited genetic variation in the contents of phytosterols when cultivated at the same location during one year (Ruibal-Mendieta et al. 2004; Iafelice et al. 2009).

The composition of phytosterols also varied depending on genotype. Chen et al. (2009) observed a significant cultivar effect in the contents of sitosterol, stigmasterol and campesterol within locations when the three bread wheat cultivars were grown at three locations in the state of Oklahoma (USA) in 2005. Significant genetic variation was also suggested in the contents of individual sterol compounds in bread wheat lines cultivated in France in 2006 (Alignan et al. 2009). In addition, the results of one study indicated significant differences in the phytosterol compositions of bread wheat, durum wheat, spelt and emmer wheat depending on genotype and species (Iafelice et al. 2009).

Table 7. Studies on genetic variation occurring in the phytosterol content of wheat, grown at a single location during one year. Total phytosterol contents are presented as µg/g of either fresh weight (FW) or dry matter (DM).

Wheat type and origin	Number of genotypes	Location	Harvesting year	Phytosterol content		Reference
Bread wheat						
Finnish cultivars	2	Finland	1997	665–715	FW	Piironen et al. 2002
European winter wheat cultivars	5	Belgium	2002	622–655	FW	Ruibal-Mendieta et al. 2004
European cultivars and synthetic populations	23	France[a]	2006	494–796	DM	Alignan et al. 2009
Cultivars, origins not reported	3	Oklahoma, USA[b]	2005	196–355	FW	Chen et al. 2009
Italian cultivars	5	Italy	2002	600–677	DM	Iafelice et al. 2009
Durum wheat						
Italian cultivars	5	Italy	2002	737–840	DM	Iafelice et al. 2009
Spelt						
European cultivars, landraces and breeding lines	16	Belgium	2002	544–807	FW	Ruibal-Mendieta et al. 2004
European cultivars	12	Italy	2002	628–819	DM	Iafelice et al. 2009
Emmer wheat (Dicoccon)						
Italian cultivars	9	Italy	2002	683–957	DM	Iafelice et al. 2009

[a] cultivated in organic conditions
[b] cultivars grown at three locations

Beyond wheat, genetic variation has been also observed in phytosterol contents of other cereal grains, e.g., oat, barley and rye. The phytosterol contents of seven Swedish oat cultivars varied significantly (Määttä et al. 1999), whereas only limited genetic variation was found in sterol contents of five oat cultivars during the HEALTHGRAIN diversity screen (Shewry et al. 2008). Nyström et al. (2008) reported a somewhat wider genetic variation in the total phytosterol contents of 10 rye varieties than the other studies that included 7 to 10 rye varieties (Piironen et al. 2002; Zangenberg et al. 2004) and only found modest variation in sterol composition. However, Zangenberg et al. (2004) demonstrated a significant genotype effect on the sterol composition of rye. The contents of phytosterols

and other phytochemicals were affected by genetic factors in barley (Andersson et al. 2008).

Steryl ferulates

The genetic variation of wheat steryl ferulates has scarcely been studied. Seitz et al. (1989) observed that the total content of steryl ferulates varied from 62–123 µg/g in bread wheat varieties, including six winter types and one spring type. The highest steryl ferulate content was found in the spring wheat genotype. The steryl ferulate compositions of genotypes varied slightly (see table 4). Hakala et al. (2002) reported nearly identical total steryl ferulate contents (62 to 63 µg/g DM) and compositions in two wholegrain wheat samples. The origins or the growing conditions of the genotypes included in these trials were not reported in detail.

Although not much is known about the effect of genetic variation in wheat, several studies have suggested that the steryl ferulate contents of rice and corn vary depending on genotype (Singh et al. 2000; Bergman and Xu 2003; Heinemann et al. 2008). More research is needed to discover if genotype also affects the content and composition of wheat steryl ferulates.

2.3.2 Environmental variation

Wheat genotypes cultivated at different environments are exposed to different growing conditions (e.g. location, year, soil properties), climatic conditions (amount of precipitation, temperature) and agronomic practices (irrigation, fertilisation). The growing environment thus provides numerous variables, which may possibly affect the phytochemical content and other characteristics of wheat. Landraces are genotypes, which have adapted to their traditional growing environments over the decades or centuries.

Phytosterols

The effects of environmental factors on wheat phytosterols have not been extensively studied. According to Chen et al. (2009), both growing location and genetic background affected the phytosterol content and composition of three wheat cultivars, which were cultivated at three locations in Oklahoma, USA, during one season in 2005. The total phytosterol contents of these cultivars varied at most from 196 to 355 µg/g FW among locations; the authors concluded that agronomic practices, climate and genotype significantly affected the wheat grain components. At one of the locations, they cultivated the genotypes both in dryland and irrigated conditions. Irrigation seemed to result in lower

phytosterol content within one cultivar, since its total phytosterol content decreased from 315 µg/g (dryland) to 202 µg/g (irrigated). Such a wide variation was not seen within the other two cultivars, indicating that irrigation may have different effects on different cultivars.

The significance of growing year in levels of phytosterols in wheat is not known. Alignan et al. (2009) compared two sowing dates during one growing season: conventional sowing in November 2005 and delayed sowing in January 2006 at one location in France. The sowing date did not affect the total phytosterol content of wheat cultivars. However, the contents of campesterol and sitostanol were affected, suggesting that the phytosterol composition may vary depending on the sowing date. The authors stated that this variation probably occurred due to differences in weather conditions (temperature and rainfall) during grain filling. The genotype effect was, however, found to be the main factor to cause variation in the phytosterol content.

Little is known about the environmental variation of phytosterols in cereals other than wheat. No locational variation was observed in the sterol contents of seven oat cultivars grown at three sites in Sweden in 1996 (Määttä et al. 1999). In two soybean varieties cultivated in various parts of Japan, growing location was shown to affect the total phytosterol content, but not the composition of sterols (Yamaya et al. 2007). Higher phytosterol contents were observed when soybean seeds were grown in warm areas. On the other hand, Zangenberg et al. (2004) found significant year-to-year variation in the total content and composition of rye phytosterols, and the warm and dry growing season was characterised by a low content of phytosterols. The environmental variation of phytosterols in five rye genotypes has been studied in the HEALTHGRAIN project (Shewry et al. 2010b), with the results indicating low variation among growing years and locations.

The data obtained in studies currently available indicate that environmental conditions may have an impact on phytosterol contents; therefore, environmental variation in wheat and other cereals needs to be investigated to a greater extent.

Steryl ferulates

There are no studies available of the environmental variation in wheat steryl ferulates. Previous research has mainly focused on steryl ferulates in rice (i.e. γ-oryzanol) and corn. Bergman and Xu (2003) studied the environmental variation of rice steryl ferulates in seven Southern U.S. rice cultivars grown at four locations and harvested in two consecutive years (1999–2000). They found that year, location and genotype had a

significant effect on levels of γ-oryzanol in rice. The steryl ferulate composition and total γ-oryzanol content of European brown rice cultivars also varied depending on the environmental conditions, both on growing year and location (Miller and Engel 2006). Further, the authors suggested that the degree of maturity did not have an effect on the steryl ferulates of rice grains. Moderately elevated growth temperature resulted in increased γ-oryzanol content when six rice lines were grown at temperature-gradient greenhouses, either at ambient or at slightly higher temperatures (Britz et al. 2007). The main steryl ferulates of the γ-oryzanol mixture were also affected by temperature. Six corn hybrids grown at various locations in the USA showed significant variation in steryl ferulate contents depending on the location of growth (Singh et al. 2000). Therefore, growing year, location and temperature tend to have an effect on levels of steryl ferulates in rice and corn.

Despite the lack of knowledge of the environmental variation of wheat steryl ferulates, the previous studies of rice and corn suggest that environmental variations do occur in cereals and possibly also in wheat. This concept, however, needs to be established by further research.

2.3.3 Variation within wheat kernel

Within the wheat kernel, starchy endosperm and germ are surrounded by bran tissues (Figure 7). Bran comprises the outer layers of the wheat kernel, which is a composite of multiple layers: the outer pericarp, intermediate layers and the aleurone. The intermediate layers are further composed of several tissues: the inner pericarp (with intermediate, cross and tube cells), the seed coat (testa) and the nucellar epidermis (hyaline layer) (Anson et al. 2012). The relative proportions of various grain tissues may vary depending on the genotype (Barron et al. 2011). The endosperm comprises approximately 80 to 85%, germ 3%, aleurone cells 6–8%, intermediate layers 3% (including the testa at 1%) and the outer pericarp 3 to 4% of the wheat kernel. The main function of the bran layers is to protect the seed (Anson et al. 2012).

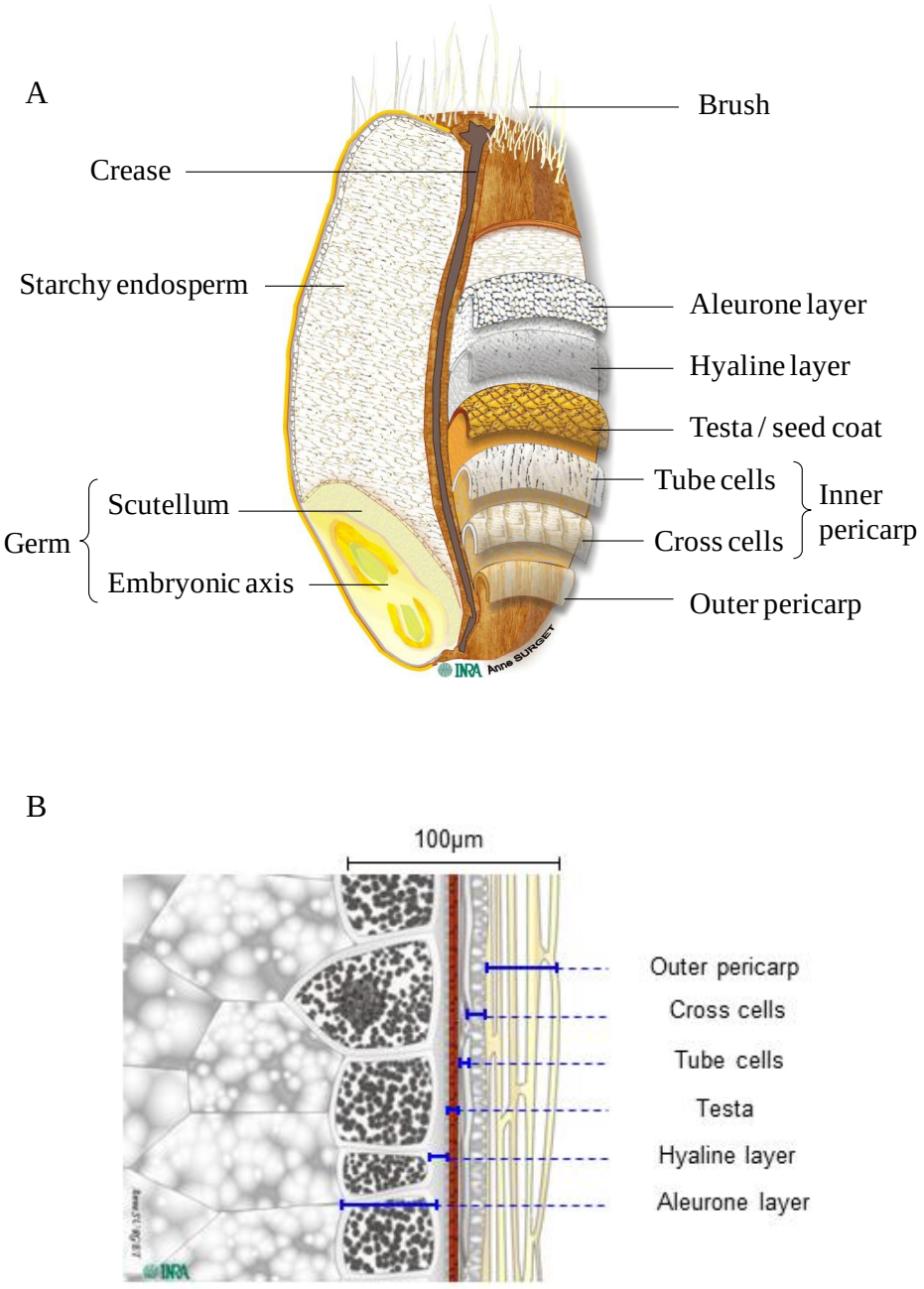

Figure 7. Histological compositions of A) whole wheat kernel and B) wheat bran. The figures are adapted with permission from Surget and Barron (2005).

Previous studies have indicated that phytosterols accumulate in the wheat bran and germ (Table 5) and steryl ferulates in the bran (Table 6). Phytosterols are also found in the inner parts of the kernel, i.e., the endosperm, because they are present in the intracellular membranes of all plant cells (Moreau et al. 2002). Steryl ferulates are concentrated in the outer layers of the wheat kernel, possibly because ferulic acid is found in the bran, with the highest levels in the aleurone and hyaline layers (Parker et al. 2005; Barron et al. 2007). The esterification of sterols within the bran layers may occur in those cells where ferulic

acid is available. The synthesis of ferulic acid is known to occur in the cytoplasm, and in wholegrain wheat, approximately 1% of ferulic acid is in free form, 7% is conjugated and 92% is bound to cell wall polysaccharides (Li et al. 2008; Saulnier et al. 2012). However, the biosynthetic pathway of steryl ferulates is not yet known. The concentration of steryl ferulates in the bran may be related to its function in the kernel, possibly a protective role against environmental stress (Seitz 1989; Britz et al. 2007).

Although the accumulation of sterols and steryl ferulates in the wheat bran is suggested, a more specific distribution of sterol compounds within the bran layers is not clearly demonstrated. Aleurone preparations with varying purity were characterised by high sterol and steryl ferulate contents, suggesting that phytosterol compounds could be concentrated in the aleurone layer of the wheat kernel (Buri et al. 2004a). Earlier, steryl ferulates were found in the wheat fraction composed of aleurone tissue and inner pericarp tissue, whereas the outer pericarp tissue did not contain steryl ferulates, which indicates an uneven distribution within the bran layers (Seitz 1989). In barley, phytosterols have been detected in the testa, a thin layer between the inner pericarp and hyaline layers (Briggs 1974). Existing studies do not show with certainty if phytosterols and steryl ferulates are accumulated in a specific tissue or layer of the wheat bran. Furthermore, no studies are available to indicate if the composition of sterol compounds varies in different bran tissues.

3 AIMS OF THE STUDY

Cereals are an important dietary source of natural phytosterols and steryl ferulates. The intake of these health-promoting compounds could be enhanced by the selection of phytosterol-rich cereal types and fractions to be exploited in cereal foods. The overall objective of this thesis was to examine the extent of variation occurring in the content and composition of phytosterols and steryl ferulates in wheat caused by genetic and environmental factors and technological dry fractionation processes. The focus was on bread wheat.

The aims of the individual studies were:

1. To study the effects of genotype and growing environment on the composition and total content of phytosterols in wheat. Various wheat genotypes cultivated in several years and locations were examined (**I**, **II**).

2. To study the effects of genotype and growing environment on the composition and total content of steryl ferulates in wheat. Selected wheat genotypes cultivated in several years and locations were examined (**III**).

3. To study the distribution and composition of phytosterols and steryl ferulates within the wheat grain and the wheat bran layers. Wheat fractions produced using both novel and conventional dry processes were examined (**IV**).

4 MATERIALS AND METHODS

This section summarises the materials and methods presented in more detail in the original publications (**I**-**IV**).

4.1 Materials

4.1.1 Cereal materials

The cereal materials were obtained from the HEALTHGRAIN project partners. All samples were stored in sealed plastic bags in the dark at -18 ºC. A sub-sample of in-house reference material was taken monthly from the freezer and stored in a desiccator at +4ºC. Before weighing for analysis, the samples and reference material were placed in the desiccator at room temperature for one hour.

Wheat genotypes grown in Hungary in 2005 (I)

As a part of the HEALTHGRAIN diversity screen, 175 wheat genotypes were studied. The bread wheat genotypes (*Triticum aestivum var. aestivum*) included 130 winter wheat types and 20 spring wheat types. Ten durum wheat (*T. turgidum var. durum*), 5 spelt (*T. aestivum var. spelta*), 5 early cultivated einkorn wheat (*T. monococcum var. monococcum*) and 5 early cultivated emmer wheat (*T. turgidum var. dicoccum*) genotypes were also included in the trial.

Genotypes differed in their geographic origin, modernity[1], type and end-use quality, e.g. texture, colour and protein content (Ward et al. 2008). The word "genotype" is used in this thesis to collectively describe landraces, breeding lines, germplasms and cultivars included in the studies. Bread wheat genotypes originated from 26 countries and were released from 1842 to 2004 (Table 8). Unreleased landraces and breeding lines were also included. Durum wheat genotypes originated from Austria (2 genotypes), Bulgaria (1), France (1), Germany (1), Hungary (1), Italy (1), Mexico (1), Russia (1) and Turkey (1). Spelt genotypes came from France (3), Germany (1) and Switzerland (1), and einkorn and emmer wheat genotypes were from France (1) and Hungary (9). A more detailed list with the names of genotypes is given in Supplementary table 1 (Appendix).

1 Modern varieties are acceptable based on the criteria of the International Union for the Protection of New Varieties of Plants (UPOV)/ distinctness, uniformity and stability (DUS) system. Old varieties do not meet the criteria of the UPOV/DUS system, (i.e. genetically heterogenous varieties). See www.upov.org.

Table 8. Origins and release years of 150 bread wheat genotypes

Origin	Release year	Modern genotypes[a]	Old genotypes[a]	Other genotypes[ab]	Total number of genotypes
Argentina	1992–1995	2			2
Australia	1983–1999	1	4		5
Austria	1985–1989		4		4
Bulgaria	1970–1982	1	2		3
Canada	1842–1972		6		6
China	1988–1989[c]	1		2	3
Croatia	1979	1			1
Czech Republic	1985		1		1
France	1940–1998	15	2	4	21
Germany	1975–2004	6	4	1	11
Hungary	1935–2002[c]	3	4	1	8
Israel	1971			1	1
Italy	1938–2003	11	4		15
Korea	1936		2		2
Mexico	1950–1993	5			5
Netherlands	1990		1		1
New Zealand	1993	1			1
Poland	1978–1997	2	1		3
Romania	1975–1991		3		3
Russia	1911–2003	2	4		6
Serbia	1967–1990	6	1		7
Switzerland	1981–1991	1	2		3
Turkey	1970–1995		4		4
Ukraine	1929–1990[c]	2	4	1	7
United Kingdom	1971–2004	10	1	1	12
United States	1948–2000[c]	7	6	2	15

[a] See Ward et al. (2008)
[b] Other genotypes include e.g. unreleased genotypes
[c] Unreleased landraces and breeding lines are also included

All wheat genotypes were grown in experimental fields of the Agricultural Research Institute of the Hungarian Academy of Sciences, at Martonvásár, Hungary. They were sown in two replicate plots in 2004 (winter types) or 2005 (spring types) and harvested in 2005. Wheat grains from the two replicate plots were combined and milled in a laboratory mill with a 0.5 mm sieve to give wholegrain flours (Rakszeki et al. 2008). Grain characteristics determined after harvesting are presented in Table 9. Ward et al. (2008) and Rakszeki et al. (2008) have described the HEALTHGRAIN study cultivation and processing conditions in more detail.

Table 9. Characteristics of the wheat genotypes included in this study.

	Number of genotypes	Moisture content (%)[a]	Bran Yield (%, FW)[a]	TKW (g/1000 kernels, FW)[a]	Lipids (%, DM)[b]
Winter wheat	130	10–13	20–33	29–56	2.0–3.5
Spring wheat	20	10–13	20–31	28–46	2.5–3.3
Durum	10	9–12	24–31	35–49	2.9–3.7
Spelt	5	9	26–28	nd[c]	3.0–3.7
Einkorn	5	8–9	21–27	nd	4.0–4.7
Emmer	5	7–9	21–24	nd	2.7–3.5

[a] Data provided by the Agricultural Research Institute of the Hungarian Academy of Sciences (Martonvásár, Hungary)
[b] Data provided by the Institute of Plant Breeding and Acclimatization (Radzikow, Poland)
[c] not determined

The 175 genotypes of the diversity screen were analysed for their phytosterol content, whereas a selection of 24 bread wheat genotypes were analysed for their steryl ferulate content.

Wheat genotypes grown in various environments in 2005-2007 (II, III)

Twenty three bread wheat genotypes (21 winter and 2 spring wheat lines) analysed in the diversity screen in 2005 were selected for further studies (See Appendix: Supplementary table 1). Three additional winter wheat genotypes that were used as standard cultivars in the HEALTHGRAIN project were included in 2006-2007 (MV Emese) or 2007 (Crousty and Tiger). The selected genotypes originated from 10 countries: Germany (5 genotypes), the United Kingdom (8), France (5), Hungary (2), Italy (1), the Netherlands (1), Russia (1), Ukraine (1), the USA (1) and China (1). The release dates of the genotypes varied from 1940 to 2003 and two of them were unreleased lines (unregistered land race and breeding line). The characteristics of wheat genotypes are listed by Shewry et al. (2010a).

The genotypes were grown in experimental fields at Martonvásár (Hungary) over three consecutive years, from 2005 to 2007. In 2007, the genotypes were also cultivated in three other sites in Europe: Enchantillon[2] (France), Woolpit[3] (United Kingdom) and Choryn[4] (Poland). As an exception, only winter wheat genotypes were grown in Poland. The agronomic practices were strictly controlled in each year and at each location. After harvesting at crop maturity, the wheat samples of the various growing years and locations were milled to ≤ 0.5-mm particle size at Martonvásár. Characteristics of growing environments and grains are given in Table 10. Details of the agronomic and weather

[2] French National Institute for Agricultural Research (INRA), Clermont Ferrand, France

[3] Nickerson Seeds, Saxham, UK

[4] Danko Plant Breeders Ltd., Choryn, Poland

conditions, soil properties, etc., at various sites are provided elsewhere (Rakszegi et al. 2008; Zhao et al. 2009; Shewry et al. 2010a).

Table 10. Characteristics of the growing environments of wheat genotypes and grain characteristics measured after harvesting.

		Martonvásár		Choryn	Woolpit	Enchantillon
Country	Hungary	Hungary	Hungary	Poland	UK	France
Year	2005	2006	2007	2007	2007	2007
Number of genotypes	24	24	26	24	26	26
Characteristics of location						
Longitude	18° 49´ E	18° 49´ E	18° 49´ E	16° 46´ E	0° 64´ E	3° 04´ E
Latitude	47° 21´ N	47° 21´ N	47° 21´ N	52° 26´ N	52° 25´ N	45° 46´ N
Altitude (m)	150	150	150	66	70	334
Precipitation (mm) [a]	116.0	128.2	126.6	101.4	232.6	204.2
Temperature (°C) [b]	19.4	19.3	20.5	17.7	14.7	18.4
Characteristics of grains						
Moisture content (%)[c]	10–13	10–12	10–12	7–11	9–14	11–12
Bran yield (%, FW)[c]	22-33	23–42	21–28	21–26	21–30	21–29
TKW (g/1000 kernels, FW)[c]	28–53	30–47	26–49	33–51	29–53	36–61
Lipids (%, DM)[d]	2.4–3.5	2.4–3.2	2.4–3.1	2.3–3.0	2.2–3.1	2.1–3.0

[a] Total precipitation from heading to harvest
[b] Mean temperature from heading to harvest
[c] Data provided by the Agricultural Research Institute of the Hungarian Academy of Sciences (Martonvásár, Hungary)
[d] Data provided by the Institute of Plant Breeding and Acclimatization (Radzikow, Poland)

Year-to-year variation in the phytosterol and steryl ferulate contents of wheat lines was studied in 24 wheat genotypes (MV Emese included) grown in Hungary during 2005-2007. All 26 genotypes grown at four locations during 2007 were analysed for phytosterols, whereas a selection of 10 wheat genotypes was analysed for their steryl ferulate content.

Wheat grain and bran fractions (IV)

The starting material in the dry fractionation processes was wholegrain wheat (var. Tiger) harvested in Germany in 2006. The coarse wheat bran was produced from the whole grains by the Department of Safety and Quality of Cereals, Max Rubner-Institut, Federal Research Institute of Nutrition and Food (Detmold, Germany). The wholegrain wheat was also analysed as flour, milled to the particle size of ≤ 0.5 mm at Martonvásár (Rakszegi et al. 2008). The wheat grain and bran fractions were produced in three various dry fractionation processes, i.e., conventional debranning and aleurone separation and a novel electrostatic separation process (Hemery et al. 2009, 2011). The grain tissue proportions of

the wheat fractions were determined using the chemical markers method, and the dry matter contents were analysed using the AACC method at the French National Institute for Agricultural Research (INRA, Montpellier, France) (Hemery et al. 2009, 2011).

Debranning process

Wholegrain wheat was fractionated in a debranning process by Bühler A.G. (Uzwil, Switzerland) using a patented method (Eugster and Gerschwiler 2006). In the debranning process, approximately 3.5% of the outermost layers of the wheat kernels were at first peeled off and the remaining kernels were milled to yield either 100% flour (100% extraction of the peeled kernel) or 76% flour (76% extraction based on the whole kernel) and bran, as shown in Figure 8 (Hemery et al. 2009). The peeled kernels were subjected to pearling to remove an additional 3% of the outer layers and similarly milled to 100% flour or 76% flour and bran.

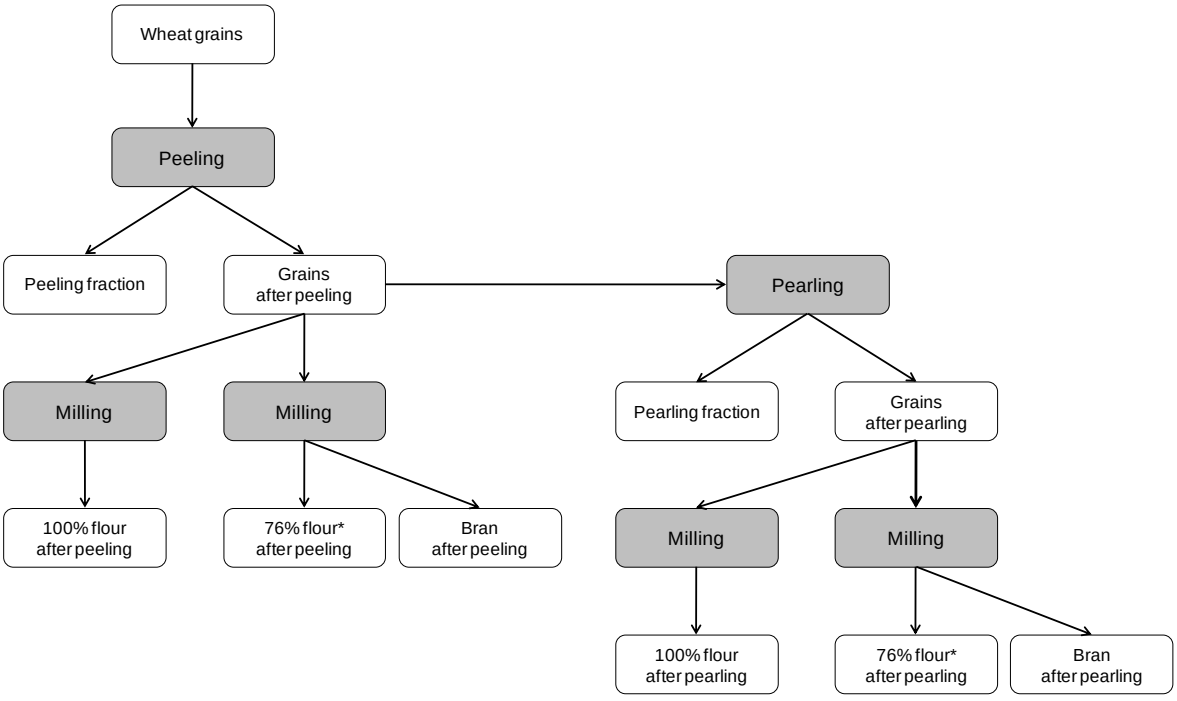

Figure 8. The debranning process of wholegrain wheat. This figure is adapted from Hemery et al. (2009), with permission. *Corresponds to 76% extraction rate flour.

Aleurone separation process

Coarse wheat bran was processed to yield two aleurone-rich fractions by Bühler A.G. using patented methods (Bohm et al. 2003; Bohm and Kratzer 2008). The bran was ground, air-classified and sieved to a standard-purity aleurone 1 (65%) and then further purified by electrostatic separation to produce an aleurone 2 fraction of higher purity (79%).

Electrostatic separation process

The wheat bran fractions, which were produced using electrostatic separation, were obtained from the INRA. The coarse bran was finely ground, and the bran particles were charged in a tribo-charging line and separated into two fractions in an electric field based on their electrical charges (Hemery et al. 2011). Three consecutive separation steps were carried out (Figure 9). In addition to the 10 bran fractions, the process yielded three deposit fractions collected from the electrodes (deposits on right and left electrodes) and the tribo-charging line (loss). The minor loss fraction was only analysed for phytosterol content due to the small amount of sample available.

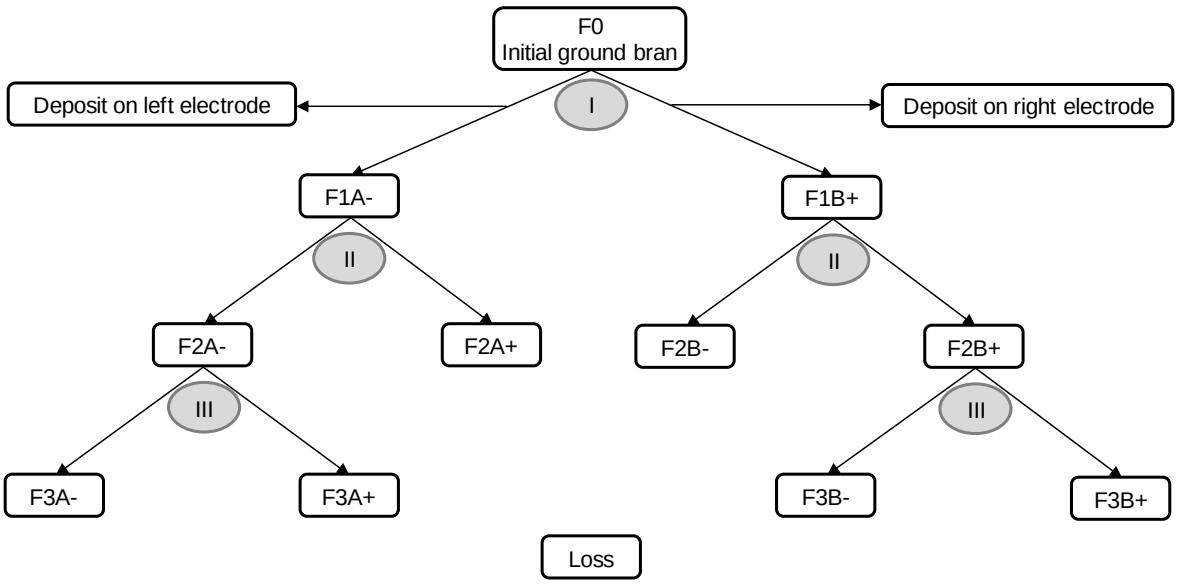

Figure 9. The electrostatic separation of wheat bran particles with three separation steps (I-III). The figure is adapted from Hemery et al. (2011), with permission.

In-house reference material (I-IV)

The winter wheat variety MV Emese was provided by the HEALTHGRAIN project to be used as an in-house reference material. Two batches of the wholegrain flour reference, harvested and milled to ≤ 0.5-mm particle size at Martonvásár (Hungary) in 2005 and 2007, were used during the experiments.

4.1.2 Standards

Dihydrocholesterol (DHC, purity 95%) was used as an internal standard in the gas chromatographic determination of phytosterols in studies **I**, **II** and **IV** and was purchased from Sigma (St. Louis, MO, USA). The standards used in the method validation (**I**) were sitostanol (purity 96%) obtained from Sigma and stigmasterol (purity 95%) obtained from Fluka Chemie (Buchs, Switzerland). Cycloartenyl ferulate (CAF), used as an external standard in the steryl ferulate analyses and for method validation in studies **III** and **IV**, was kindly provided by Dr. Parkash Kochhar (Good-Fry International, Rotterdam, The Netherlands).

4.2 Experimental

4.2.1 Analysis of phytosterols using gas chromatography (I, II, IV)

Sample preparation

The procedure used for phytosterol analysis included acid and alkaline hydrolyses and was based on the method of Piironen et al. (2002). The internal standard (DHC, 40 µg) was first added into a 0.5-gram cereal sample. The sample was then subjected to acid hydrolysis with hydrochloric acid (HCl) to liberate sterols from their glycosidic conjugates. After acid hydrolysis and the extraction of lipids, alkaline hydrolysis with potassium hydroxide (KOH) saponified the lipids and hydrolysed the esterified sterols into free sterols. The unsaponifiable lipids (containing free sterols) were extracted into cyclohexane and purified by solid-phase extraction (SPE) using silica cartridges (Strata SI-1, 500 mg, Phenomenex, Torrance, CA, USA). Prior to the gas chromatographic analysis, phytosterols were derivatised to trimethylsilyl (TMS) ethers using N,O-bis(trimethylsilyl) trifluoroacetamide (BSTFA, Merck, Darmstadt, Germany) and trimethylchlorosilane (TMCS, Fluka Chemie) in a ratio of 99:1 (v/v) as the reagents in anhydrous pyridine (Merck). Each sample was analysed in duplicate.

Gas chromatographic analysis

Phytosterols were analysed using gas chromatography with flame ionisation detection (GC-FID, 6890N GC, Agilent Technologies, Santa Clara, CA, USA). The GC was equipped with a Crossbond® 5% diphenyl – 95% dimethyl polysiloxane capillary column (RTX-5w/Integra-Guard, 60 m × 0.32 mm, film thickness 0.10 µm, Restek Corp., Bellefonte, PA, USA), as reported previously (Piironen et al. 2002).

Fifteen individual phytosterol species were identified by comparison to the relative retention times of commercial standards, data obtained with gas chromatography-mass spectrometry (GC-MS) and literature data (Kamal-Eldin et al. 1992; Goad and Akihisa 1997; Piironen et al. 2002). The GC-MS method is described by Piironen et al. (2002). The 4-desmethyl sterols analysed were sitosterol, sitostanol, campesterol, campestanol, brassicasterol, stigmasterol, Δ5-avenasterol, Δ7-avenasterol, stigmasta-5,24(25)-dienol, and Δ7-stigmastenol. The 4-monomethyl sterols (citrostadienol and gramisterol) and 4,4'-dimethyl sterols (cycloartenol, 24-methylenecycloartanol and α-amyrin) were also identified. Phytosterols were quantified using DHC as an internal standard.

Quality assurance

For the analysis of phytosterols in cereal samples in studies **I**, **II** and **IV**, the method described and validated by Piironen et al. (2002) was modified, e.g. by scaling the sample size to half. To ensure the performance of the modified method, the recoveries for sitostanol and stigmasterol standards added to the wholegrain wheat flour were determined (**I**), and the repeatability was evaluated by reanalysing the in-house reference flour (**I**, **II**). According to a t-test ($p < 0.05$), the modified method gave similar results as the original method (**I**).

For daily quality control, the in-house reference flour was included in each series of samples. Reanalysis of the sample series was required if the phytosterol content of the reference flour was found to exceed the action limits determined in studies **I** and **II**. In addition, to confirm the performance of the GC-FID, the peak intensities and relative retention times of dihydrocholesterol, cholesterol and stigmasterol were followed daily by analysing a mixture of these sterol standards. The limit of determination for individual sterol compounds in the GC-FID analysis of cereal samples was verified (**I**). Cereal samples were analysed for the phytosterol content in duplicate. If the difference between duplicate results exceeded 5%, the sample was reanalysed and the mean of all replicate results was calculated and reported. Possible outliers of the replicate results were identified using Dixon's Q-test ($p < 0.05$) and rejected.

4.2.2 Analysis of steryl ferulates using high-performance liquid chromatography (III, IV)

Sample preparation

The steryl ferulates were extracted and purified from cereal samples using the procedures of Hakala et al. (2002) and Nyström et al. (2007) based on the method of Seitz (Seitz 1989). The steryl ferulates were extracted from the 2-gram cereal sample with hot acetone under reflux using a Soxtec Avanti 2050 apparatus (Foss Tecator Ab, Höganäs, Sweden) at 150°C (Nyström et al. 2007). As an exception, the bran fractions were extracted as 1-gram samples. The extract was evaporated to dryness and dissolved in methanol. During the following base-acid purification (Hakala et al. 2002), KOH was first added, and the neutral lipids were removed by washing with heptane under alkaline conditions. Next, HCl was added, and the steryl ferulates were extracted with heptane in acidic conditions. Finally, steryl ferulates were transferred to a mixture of methanol and water (98:2, v/v) prior to liquid chromatographic analysis. Each cereal sample was analysed in triplicate.

High-performance liquid chromatographic analysis

Steryl ferulates were analysed using reversed-phase high performance liquid chromatography (HPLC) with ultraviolet (UV) detection at 325 nm (Hakala et al. 2002). The HPLC apparatus consisted of a pump (Waters 515, Waters Corporation, Milford, MA, USA), autosampler (Waters 717 Plus) and tunable absorbance detector (Waters 486), and was equipped with a reversed-phase column (Zorbax ODS, 4.6 x 250 mm, 5 µm, Agilent Technologies) at 50ºC. The mobile phase was methanol:water:acetic acid (97:2:1, v/v/v), and an isocratic elution with a flow rate of 1.5 ml/min was used.

The identification of steryl ferulates was based on the HPLC-MS analysis described in study **III** and literature data (Hakala et al. 2002). The analysed individual steryl ferulates were campesteryl ferulate, sitostanyl ferulate and a mixture of campestanyl and sitosteryl ferulates. CAF was used as an external standard for quantification of the steryl ferulates. The concentration of the standard solution was determined daily prior to the HPLC analysis using a spectrophotometer (Lambda 25, UV/VIS Spectrometer, Perkin Elmer, Shelton, CT, USA) at 325 nm, as described by Hakala and colleagues (2002).

Quality assurance

The performance of the analytical method was verified with recovery and repeatability studies prior to analysis of steryl ferulates in the cereal samples in studies **III** and **IV**. The recovery of CAF standard added to the in-house reference flour was determined, and the

repeatability of the method was followed by analysing the in-house reference daily (**III**). The limits of detection and quantification and the linearity of the detector response for HPLC-UV were determined using the CAF standard solution (**III**).

The in-house reference flour was analysed in each extraction series. (**III**, **IV**). It was ensured that the steryl ferulate content of the in-house reference was within the action limits defined in study **III**; otherwise, the analysis of the sample series was repeated. Cereal samples were analysed for the steryl ferulate content in three replicates. A sample was reanalysed if the coefficient of variation (CV) of the three replicate results was higher than 10%. The mean of all replicate results was then calculated and reported. The possible outliers were detected with Dixon's Q-test ($p < 0.05$) and rejected.

4.2.3 Data analysis

The phytosterol and steryl ferulate concentrations are presented as averages of replicate samples on a DM basis (**I-IV**). In this thesis, all results are given on a DM basis from this section onward, if not otherwise stated.

The statistical differences between the total phytosterol contents of different wheat species in study **I** were measured with a non-parametric Kruskal-Wallis test and a notched Box-and-Whisker plot. A median test was used because data were not normally distributed (as detected by Cochran's C test). The effects of genotype, growing location and year on the contents of phytosterol compounds in wheat (**II**, **III**) were examined with a two-way analysis of variance (ANOVA) using the genotype as a block factor and the Fisher's least-significant difference (LSD) procedure. The composition data of studies **II-IV** were subjected to Principle Component Analysis (PCA) to describe the variation occurring among the wheat genotypes or fractions and to visualise the relationships among different variables. For PCA, the data were scaled variable wise to unit variance and centred to a zero mean. Furthermore, the linear correlations between the contents of the phytosterol compounds and other kernel characteristics were determined using Pearson's correlation coefficients (r) in studies **I-IV**. In all analyses, *p*-values lower than 0.05 were considered statistically significant. Statistical analyses were performed using Statgraphics Plus 4.0, Centurion XV or Centurion XVI software (StatPoint Technologies, Inc., Warrenton, VA, USA), The Unscrambler® v. 9.0 or The Unscrambler® X v. 10.1 software (Camo Software AS, Oslo, Norway) and MatLab 7.10 R2010a software (The MathWorks, Inc., Natick, MA, USA).

5 RESULTS

5.1 Phytosterols in wheat grains (I–II)

5.1.1 Effect of genotype on phytosterols in wheat

Total content of phytosterols

During the HEALTHGRAIN diversity screen study in 2005, various cereal species and genotypes were cultivated under similar environmental conditions at the same location in Hungary. Figure 10 represents the total phytosterol contents of studied wheat species, including winter and spring types of bread wheat, spelt, durum wheat and ancient einkorn and emmer wheat (**I**). The highest average phytosterol contents were found in einkorn (1054 ± 85 µg/g) and durum wheat (987 ± 79 µg/g), whereas winter wheat (841 ± 55 µg/g) and emmer wheat (857 ± 53 µg/g) were the poorest sources of phytosterols among the cereal types. On average, einkorn wheat contained 25% more phytosterols than the winter type of bread wheat.

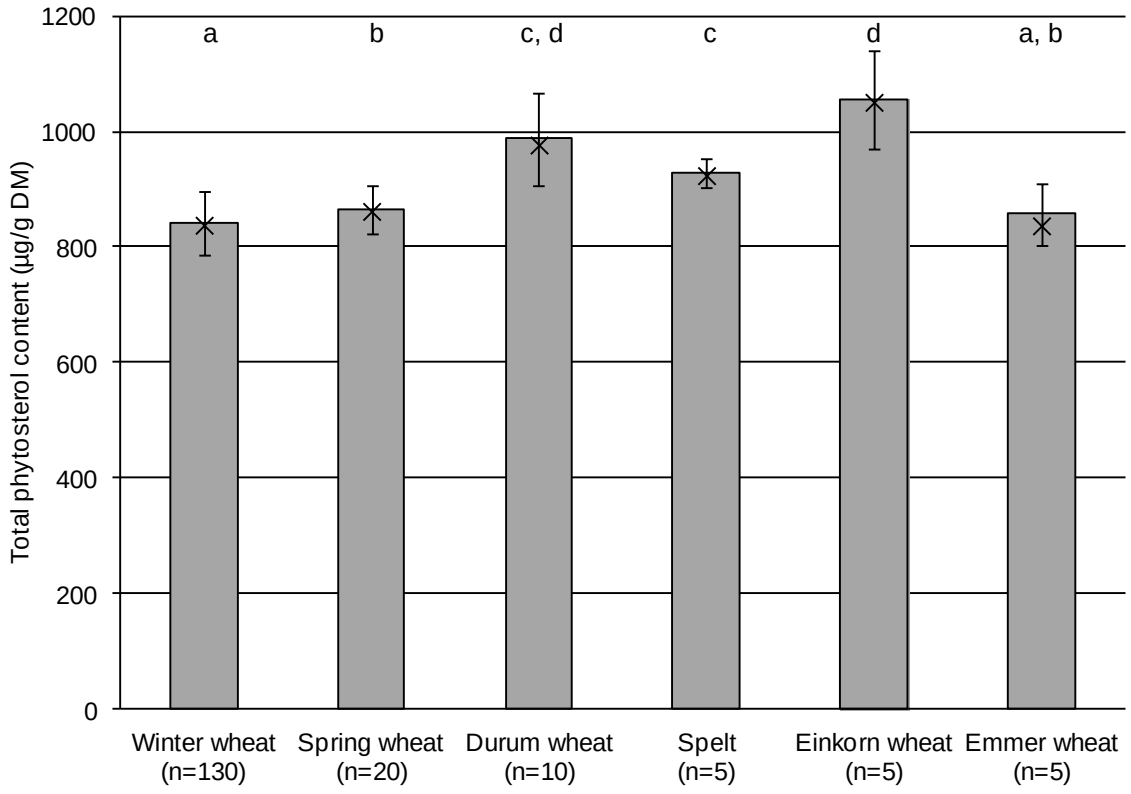

Figure 10. Phytosterol contents (mean ± standard deviation (SD), µg/g DM) of various wheat types grown in Hungary during 2005. Medians are represented by crosses (×) within the columns. Medians of the wheat types with the same superscript letter on top of the column are not significantly different ($p < 0.05$).

The widest range in the phytosterol content, indicating high variation, was observed among the winter wheat genotypes. Within bread wheat genotypes, total sterol content varied from 670–959 µg/g DM (Figure 11). The majority of the winter types (61%) and spring types (75%) of bread wheat contained 800 to 899 µg/g phytosterols (**I**; Table 2). The most phytosterol-rich bread wheat genotype, the modern English winter type variety Claire, contained 43% more phytosterols than modern French winter wheat variety Qualital, which had the lowest content. The bread wheat genotypes included both modern and old cultivars and originated from various parts of the world. However, the release year and country of origin did not have considerable effect on the total content of phytosterols (Figure 12). Among all the wheat types, the Hungarian einkorn wheat variety 08-2004 possessed the highest total phytosterol content (1187 µg/g) observed in study **I**.

Selected bread wheat genotypes grown in various environments (**II**) showed statistically significant differences ($p < 0.0001$) in their total phytosterol contents due to genetic factors. The average sterol contents of 26 genotypes grown in six environments varied from 700 ± 42 (Herzog) to 928 ± 54 µg/g (Claire).

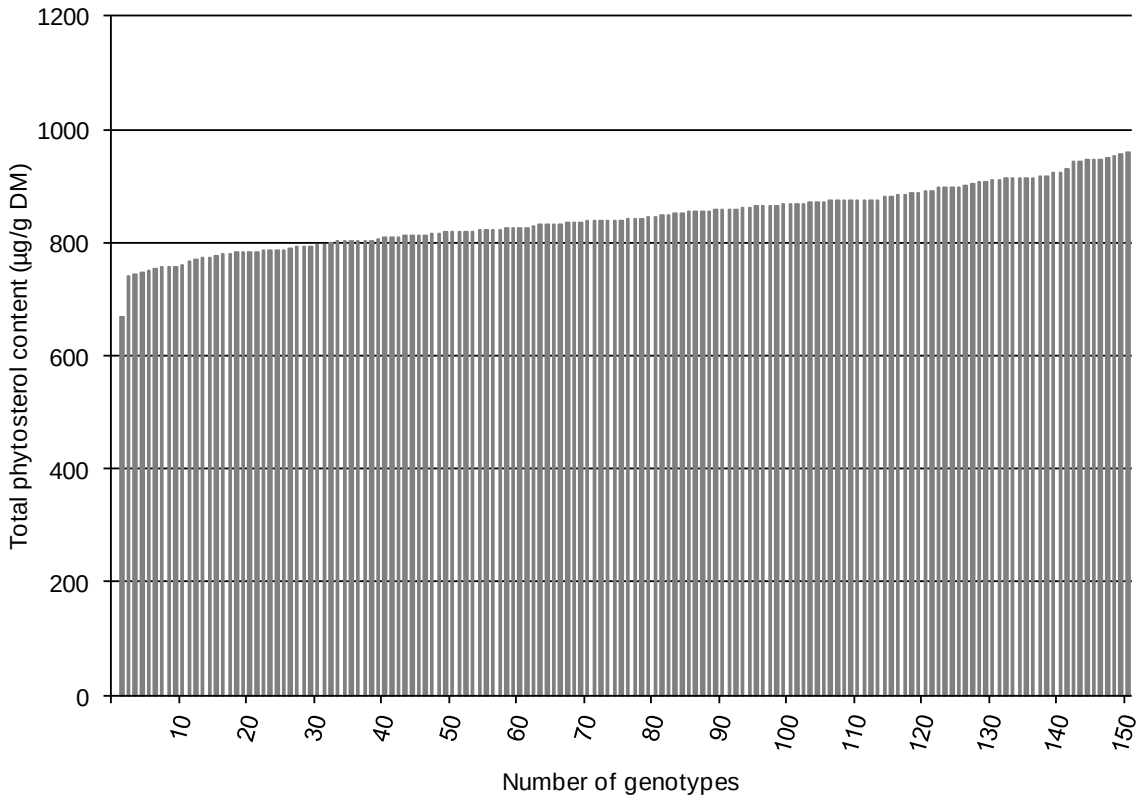

Figure 11. Total phytosterol contents (µg/g DM) of bread wheat genotypes (n=150) in order of increasing concentration.

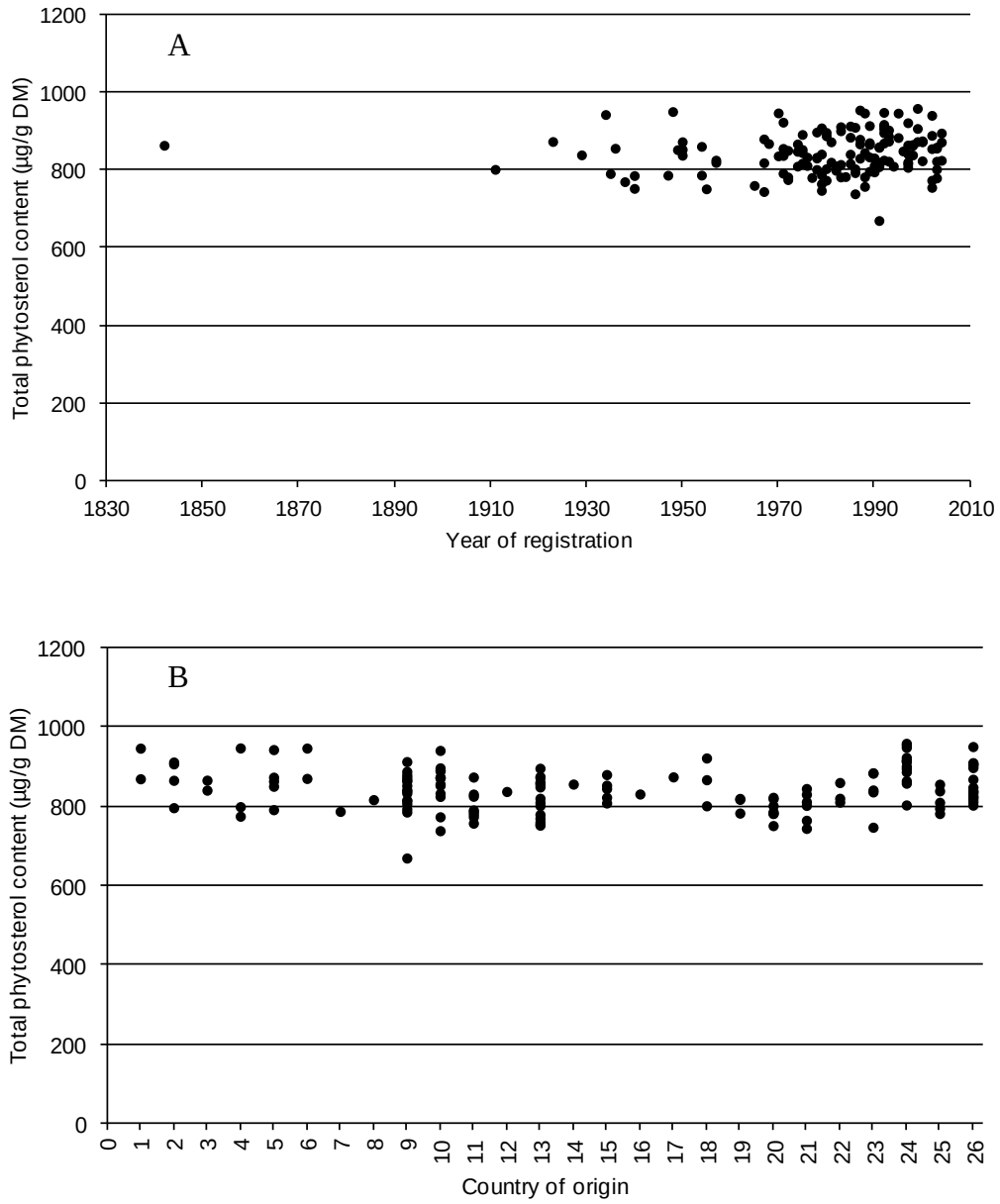

Figure 12. Relationships between total phytosterol content and **A**) year of registration (unregistered genotypes excluded) and **B**) country of origin within 150 bread wheat genotypes. The country codes are as follows: 1, Argentina; 2, Australia; 3, Austria; 4, Bulgaria; 5, Canada; 6, China; 7, Croatia; 8, Czech Republic; 9, France; 10, Germany; 11, Hungary; 12, Israel; 13, Italy; 14, Korea; 15, Mexico; 16, Netherlands; 17, New Zealand; 18, Poland; 19, Romania; 20, Russia; 21, Serbia; 22, Switzerland; 23, Turkey; 24, UK; 25, Ukraine; 26, USA.

Phytosterol composition

When comparing the different wheat types, slight differences were observed in the sterol profiles (**I**). The average phytosterol compositions of the spring and winter types of bread wheat were similar. The most abundant phytosterol species in all wheat types was sitosterol, followed by campesterol, sitostanol and campestanol. On average, the highest relative proportion of sitosterol was found in bread wheat (52%), and the lowest was in durum wheat (45%). Campesterol accounted for 15% of total sterols in bread wheat and for 14 to 18% in other wheat types. The proportion of sitostanol and campestanol in total was higher in durum, spelt and emmer wheat (on average 27% of total sterols) than in einkorn (22%) and bread wheat (24%). Other sterol species were present as minor compounds, each comprising on average from 0–3% of the total phytosterol content. In all, the total minor sterols ranged from 9 to 12% of the total sterol content.

Table 11. Phytosterol compositions of various wheat types, presented as ranges (µg/g DM). The relative contents (% of total sterols) are given in parentheses.

Wheat type	N[a]	Sitosterol	Campe-sterol	Sitostanol	Campe-stanol	Stigma-sterol	Total stanols	Total
Winter wheat	130	342–530 (47–59%)	95–169 (11–20%)	49–155 (6–19%)	48–121 (6–13%)	13–25 (2–3%)	97–263 (11–29%)	670–959
Spring wheat	20	412–527 (49–61%)	114–153 (13–17%)	57–138 (7–16%)	42–103 (5–12%)	16–24 (2–3%)	100–232 (12–26%)	797–949
Durum wheat	10	410–482 (40–48%)	126–185 (14–18%)	114–158 (12–15%)	105–187 (11–17%)	24–32 (2–3%)	223–338 (24-31%)	871–1106
Spelt	5	437–473 (47–53%)	120–143 (13–16%)	100–164 (11–18%)	81–112 (9–12%)	18–25 (2–3%)	181–277 (20–30%)	893–963
Einkorn wheat	5	454–628 (45–53%)	142–314 (15–26%)	34–155 (3–16%)	46–129 (4–12%)	13–21 (1–2%)	80–271 (7–28%)	976–1187
Emmer wheat	5	363–422 (45–46%)	128–145 (15–16%)	107–152 (13–16%)	95–118 (12–13%)	26–30 (3–4%)	201–270 (25–29%)	796–937

[a] Number of genotypes

The phytosterol compositions of individual wheat genotypes varied considerably (**I**). Among the 150 bread wheat genotypes, the relative proportion of sitosterol varied from 47 to 61%, whereas narrower ranges were observed within durum, spelt, einkorn and emmer wheat genotypes (Table 11). The proportion of campesterol from total phytosterols ranged from 11 to 20% among the bread wheat genotypes and from 13 to 26% among other wheat genotypes. The greatest variation was found in the proportion of total stanols, since the stanols comprised 11–29% and 7–28% of the total sterols within the bread wheat and einkorn wheat genotypes, respectively. In durum, spelt and emmer wheat genotypes, stanols comprised 20–31% of the total sterols. The other sterols each ranged from 0–3% and 6–13% in total of all the sterols within the bread wheat genotypes, whereas the respective values among the other wheat types were 0–5% and 9–15%.

Genetic variation in phytosterol composition was shown to be statistically significant ($p < 0.0001$) in study **II** within selected bread wheat genotypes cultivated in various environments. The PCA showed that a high total phytosterol content was associated with a low relative content of sitosterol and a high relative content of stanols (Figure 14).

5.1.2 Effect of growing environment on phytosterols in wheat

Total content of phytosterols

The average phytosterol contents of bread wheat genotypes grown in Hungary in three consecutive years did not vary significantly over the growing years, the values being 857 ± 74, 881 ± 70 and 868 ± 79 µg/g DM in 2005, 2006 and 2007, respectively (Figure 13). However, certain individual genotypes showed a wider variation among years than the others; the phytosterol content of modern British winter wheat variety Avalon ranged from 739 to 884 µg/g, whereas that of modern German winter variety Tommi only varied from 880 to 890 µg/g (**II**; Figure 1). The lowest phytosterol contents were frequently found in samples grown in 2005.

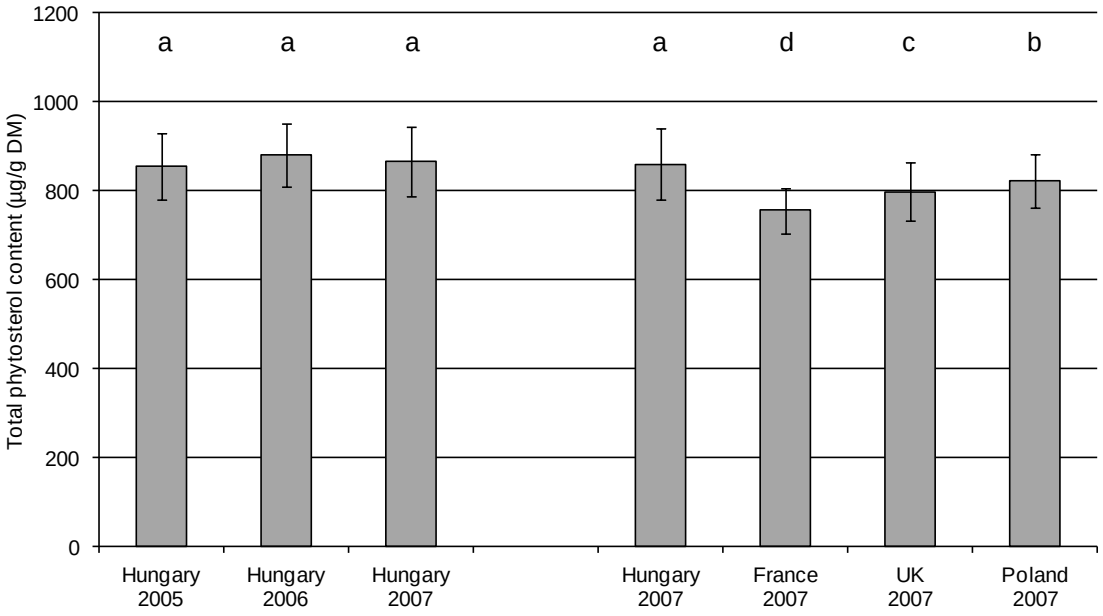

Figure 13. Phytosterol contents (mean ± SD, µg/g DM) of bread wheat lines grown in Hungary in 2005–2007 (24 genotypes) or at four locations in 2007 (26 genotypes). The mean phytosterol contents with the same superscript letter on top of the column within a subgroup are not significantly different ($p < 0.05$).

The location of growth had a significant effect ($p < 0.0001$) on the total phytosterol content of bread wheat, being greater than the effect of genetic factors among selected genotypes. The level of phytosterols differed significantly among all countries (Figure 13). This trend was also demonstrated in PCA (Figure 14). The highest average content was observed in genotypes grown in Hungary (861 ± 80 µg/g) and the lowest in France (755 ± 50 µg/g). The bread wheat genotypes grown in Poland and the United Kingdom contained on average 823 ± 61 and 798 ± 66 µg/g phytosterols, respectively. Following the same pattern, most of the individual genotypes possessed the highest sterol content when grown in Hungary and the lowest when grown in France. The extent of variation among locations differed within individual genotypes (**II**; Figure 2), and the variation was commonly wider among growing sites than among years. The widest range in phytosterol content was found in British winter wheat Riband (760–972 µg/g) and the narrowest range in Hungarian variety MV Emese (703–730 µg/g).

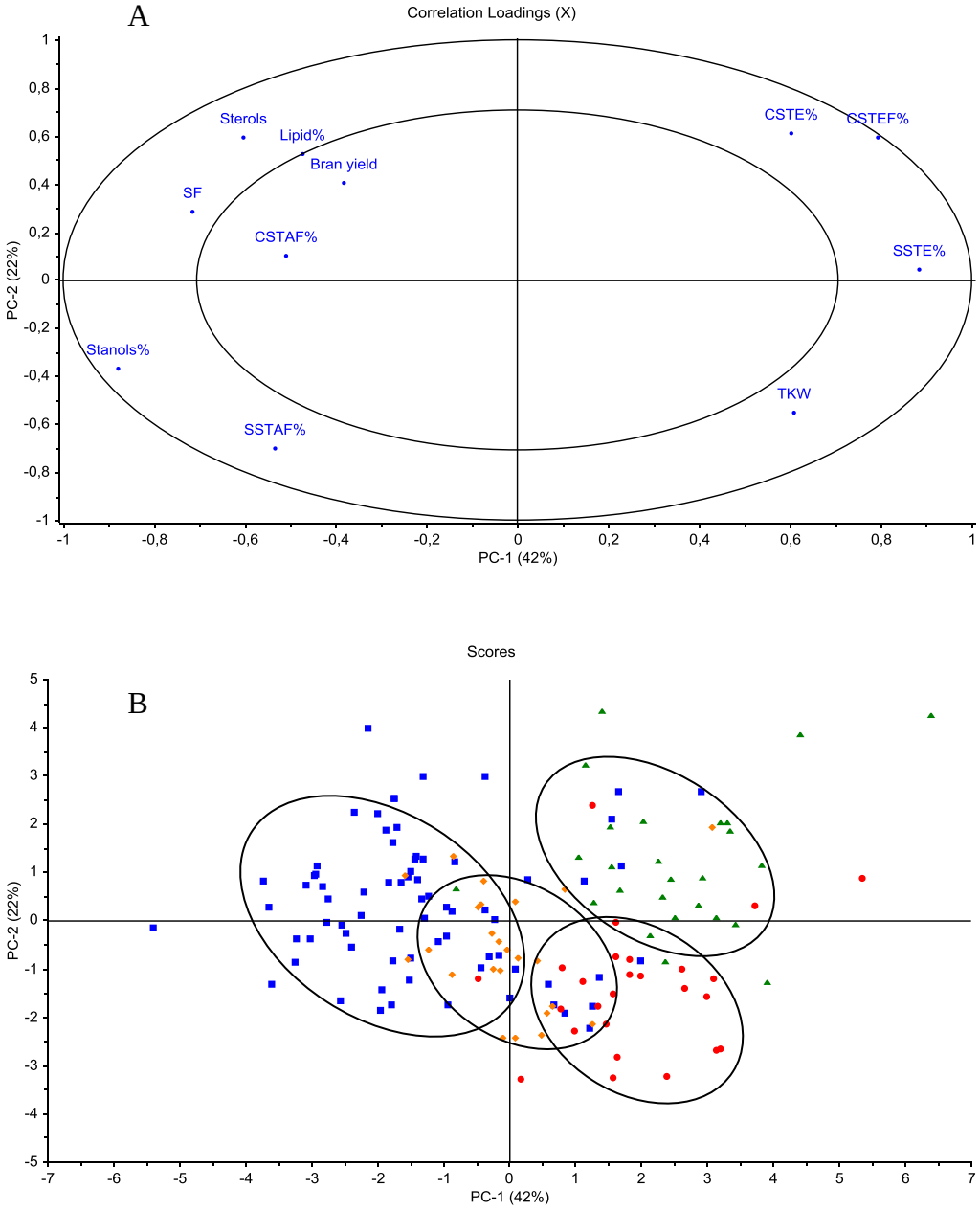

Figure 14. Principal component analysis of grain charasteristics of the wheat genotypes grown in different environments (N=150); **A**) loadings plot of the variables (sterols, total phytosterol content; SSTE%, relative content of sitosterol; CSTE%, relative content of campesterol; stanols%, relative content of stanols; SF, total steryl ferulate content; CSTAF%, relative content of campestanyl and sitosteryl ferulates; SSTAF%, relative content of sitostanyl ferulate; CSTEF%, relative content of campesteryl ferulate; lipid%, lipid content; Bran yield; TKW, thousand kernel weight); **B**) scores plot of wheat samples grouped by the growing location (■, Hungary; ♦, Poland; ●, France; ▲, United Kingdom).

Phytosterol composition

The phytosterol composition of bread wheat was affected by both growing year and location (**II**). Although the differences in average phytosterol compositions were statistically significant ($p < 0.0001$), the differences in the phytosterol profiles of individual wheat genotypes were moderate. The proportion of the major compound, sitosterol, ranged from 49 ± 2% (2006) to 52 ± 2% (2005) over the growing years, and from 50 ± 2% (Hungary) to 55 ± 1% (United Kingdom) when grown at four sites in 2007 (Table 12). The relative content of stanols varied from 24 ± 2% (2005) to 26 ± 2% (2006) among years and from 20 ± 2% (United Kingdom) to 25 ± 2% (Hungary) among locations. A low relative content of sitosterol and a high relative content of stanols were related to a high total sterol content (Figure 14). The variation within the genotypes was somewhat wider among locations than among years.

Table 12. Phytosterol compositions of wheat genotypes grown in various environments, presented as mean ± SD (µg/g DM). The relative contents (% of total sterols) are given in parentheses.

Environ-ment	N[a]	Sitosterol	Campe-sterol	Sitostanol	Campe-stanol	Stigma-sterol	Total stanols	Total
Years								
Hungary 2005	24	441 ± 38 (52 ± 2%)	136 ± 14 (16 ± 1%)	113 ± 14 (13 ± 1%)	94 ± 13 (11 ± 1%)	19 ± 3 (2 ± 0%)	207 ± 26 (24 ± 2%)	857 ± 74
Hungary 2006	24	434 ± 35 (49 ± 2%)	132 ± 13 (15 ± 1%)	130 ± 15 (15 ± 1%)	102 ± 13 (12 ± 1%)	21 ± 4 (2 ± 0%)	232 ± 25 (26 ± 2%)	881 ± 70
Hungary 2007	24	438 ± 35 (50 ± 2%)	140 ± 18 (16 ± 1%)	116 ± 14 (13 ± 1%)	99 ± 15 (11 ± 1%)	23 ± 5 (3 ± 0%)	216 ± 28 (25 ± 2%)	868 ± 79
Locations								
Hungary 2007	26	434 ± 37 (50 ± 2%)	139 ± 18 (16 ± 1%)	116 ± 14 (13 ± 1%)	99 ± 14 (11 ± 1%)	23 ± 5 (3 ± 0%)	214 ± 27 (25 ± 2%)	861 ± 80
France 2007	26	402 ± 29 (53 ± 2%)	122 ± 12 (16 ± 1%)	93 ± 10 (12 ± 1%)	77 ± 10 (10 ± 1%)	16 ± 2 (2 ± 0%)	170 ± 19 (22 ± 2%)	755 ± 50
UK 2007	26	435 ± 35 (55 ± 1%)	143 ± 16 (18 ± 1%)	82 ± 12 (10 ± 1%)	75 ± 10 (9 ± 1%)	19 ± 4 (2 ± 0%)	157 ± 19 (20 ± 2%)	798 ± 66
Poland 2007	24	427 ± 31 (52 ± 1%)	130 ± 14 (16 ± 1%)	109 ± 9 (13 ± 1%)	88 ± 9 (11 ± 1%)	19 ± 3 (2 ± 0%)	197 ± 17 (24 ± 1%)	823 ± 61

[a] Number of genotypes

5.1.3 Relating phytosterol contents with wheat kernel characteristics

PCA (Figure 14) showed that a high content of phytosterols was associated with high lipid content, a high bran yield and a low thousand kernel weight. The linear correlations with these agronomical kernel characteristics were, however, relatively weak. The significant correlations found between the total phytosterol content and the relative proportion of lipids (r = 0.4612) or bran yield (r = 0.3437) in the wholegrain flours were positive, whereas the correlation with the thousand kernel weight (r = –0.5401) was negative (combined data from studies **I** and **II**).

5.2 Steryl ferulates in wheat grains (III)

5.2.1 Effect of genotype on steryl ferulates in wheat

Total content of steryl ferulates

During the diversity screen study in 2005, in which all genotypes were grown at one location during a single year, the total steryl ferulate contents of bread wheat lines ranged from 79 to 123 µg/g DM, showing considerable variation (Figure 15). Italian old cultivar San Pastore possessed the lowest steryl ferulate content and British modern cultivar Claire was the most steryl ferulate-rich genotype. Most of the genotypes (approximately 70%) contained 90–110 µg/g steryl ferulates. Depending on the genotype, steryl ferulates accounted for 7–9% of total phytosterols and 25–42% of stanols among these wheat lines. The year of registration and the geographical origin of the wheat genotypes did not have a considerable effect on the total steryl ferulate content (Figure 16).

Genotype was shown to have a significant effect on steryl ferulate content ($p < 0.0001$) when wheat lines were grown in different environments during 2005-2007 (**III**; Table 1). The genotypes that contained the most steryl ferulates over the growing years and locations included the German and French winter wheat lines Crousty, Disponent and Tommi (with an average steryl ferulate content up to 114 µg/g), whereas the lowest contents of steryl ferulates (from 75 ± 7 µg/g) were found in the winter types Estica and San Pastore. When grown in various environments, steryl ferulates accounted for 6 to 10% of the total phytosterol content and for 24 to 43% of total stanols in wheat, depending on the genotype.

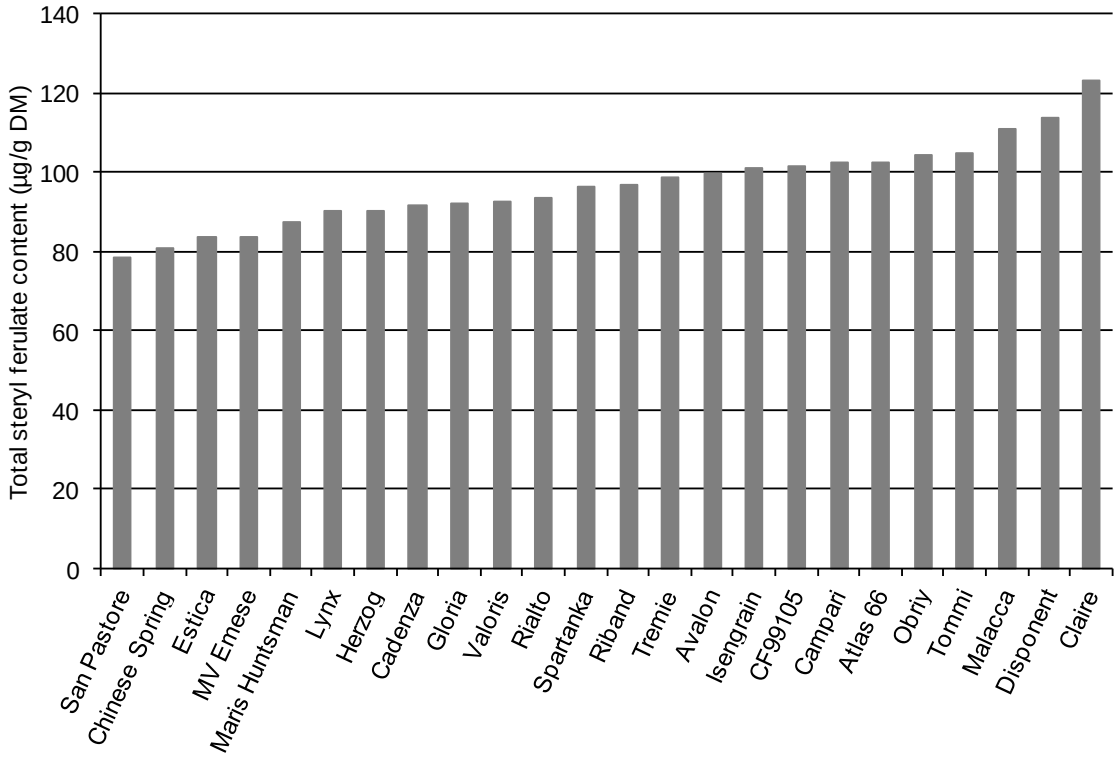

Figure 15. Total steryl ferulate contents (µg/g DM) of bread wheat genotypes (n = 24) grown at one location during the same season.

Steryl ferulate composition

The steryl ferulate composition of wheat was affected by the genotype ($p < 0.0001$). Four individual steryl ferulate species, i.e. campestanyl, sitostanyl, campesteryl and sitosteryl ferulates, were identified in all genotypes. Campestanyl ferulate was coeluted with sitosteryl ferulate, and the HPLC-MS analysis showed that campestanyl ferulate was the most abundant compound, accounting for approximately 80% of the total content of the coeluting compounds. Among genotypes grown at a single location in 2005, the relative contents of campestanyl (including sitosteryl ferulate), sitostanyl and campesteryl ferulates ranged as follows: 50–56%, 27–38% and 10–22%, respectively. Similarly, when grown in six different environments, the sum of campestanyl and sitosteryl ferulates varied on average from 50 to 58% of total steryl ferulates. Sitostanyl ferulate comprised from 24 to 36% of the total steryl ferulates in wheat genotypes. The relative content of campesteryl ferulates also varied widely among wheat genotypes, since it was 11 to 22% of the total content.

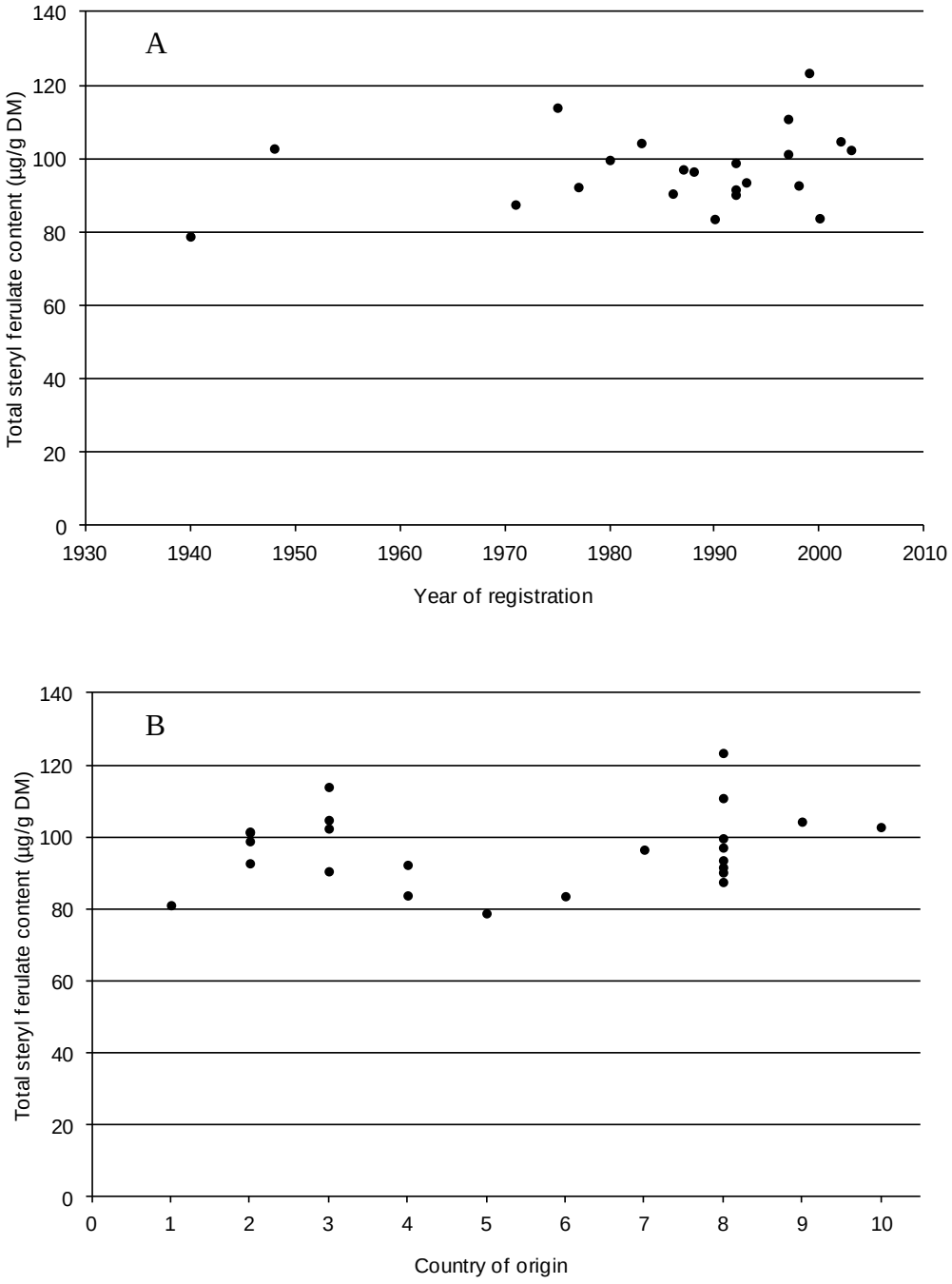

Figure 16. Relationships between total steryl ferulate content and **A**) year of registration (two unregistered genotypes excluded), and **B**) country of origin within 24 bread wheat genotypes. The country codes are as follows: 1, China; 2, France; 3, Germany; 4, Hungary; 5, Italy; 6, Netherlands; 7, Russia; 8, UK; 9, Ukraine; 10, USA.

5.2.2 Effect of growing environment on steryl ferulates in wheat

Total content of steryl ferulates

Genotypes cultivated at one location over three consecutive years did not show year-to-year variation in steryl ferulate contents (Figure 17). The average steryl ferulate content of bread wheat was at the same level in 2005 (97 ± 11 µg/g), 2006 (100 ± 12 µg/g) and 2007 (102 ±16 µg/g). The most stable genotype over the growing years was modern English variety Malacca, with a steryl ferulate content ranging from 108 to 111 µg/g (**III**; Figure 3). The genotype with the widest variation was modern French variety Valoris, which contained 93 to 125 µg/g steryl ferulates, depending on the year of growth.

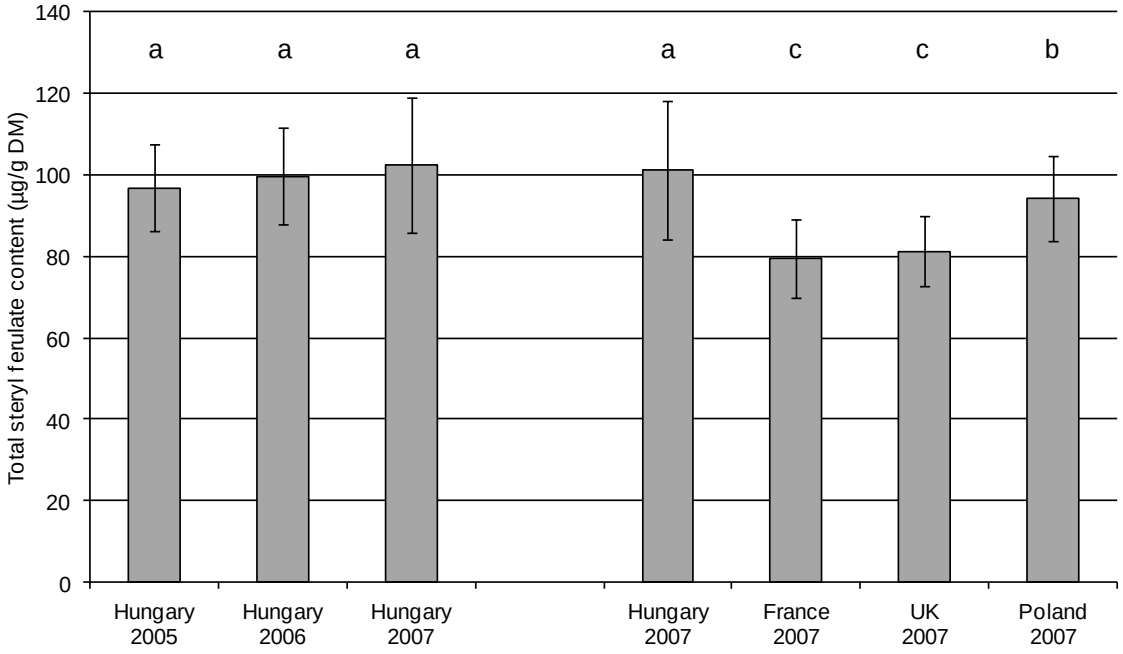

Figure 17. Steryl ferulate contents (mean ± SD, µg/g DM) of bread wheat lines grown in Hungary in 2005–2007 (24 genotypes) or at four locations in 2007 (10 genotypes). The mean steryl ferulate contents with the same superscript letter on top of the column within a subgroup are not significantly different ($p < 0.05$).

Growing the same genotypes at different locations caused significant variation ($p < 0.0001$) in total steryl ferulate contents of wheat genotypes (See also PCA in Figure 14). The location of growth had a greater effect on steryl ferulate contents than genetic factors. The lowest average contents were observed in the genotypes cultivated in France (79 ± 10 µg/g) and the United Kingdom (81 ± 9 µg/g), whereas higher content (94 ± 10 µg/g) was found in those grown in Poland (Figure 17). Wheat genotypes grown in Hungary possessed the highest levels of steryl ferulates: 101 ± 17 µg/g on average. The Dutch genotype Estica was the most stable individual genotype, containing 67 to 78 µg/g steryl ferulates

depending on the location (**III**; Figure 4). The widest variation (81–126 µg/g) was observed in the modern French genotype Tremie. Generally, the growing location caused more variation in the steryl ferulate contents of individual genotypes than the year of growth.

Steryl ferulate composition

Both the growing year and location had moderate but statistically significant effects ($p < 0.0001$) on the steryl ferulate composition. The relative content of the main compound campestanyl ferulate (including sitosteryl ferulate) was slightly higher in genotypes grown in Hungary in 2007 (on average, 56%) compared to other growing years (Table 13). Campesteryl ferulate was also showing the highest relative content in 2007, whereas the proportion of sitostanyl ferulate was significantly lower in 2007 than in the other growing years. At various growing locations, genotypes had the highest relative content of the main compound at Hungarian site, whereas at other sites, content did not differ. The relative content of campesteryl ferulate was the highest when bread wheat was grown in the UK and lowest at Hungarian and Polish sites. In contrast, the highest content of sitostanyl ferulate was observed in genotypes grown in Poland and the lowest in those grown in the UK.

Table 13. Steryl ferulate compositions of wheat genotypes grown in various environments, presented as mean ± SD (µg/g DM). The relative contents (% of total steryl ferulates) are given in parentheses.

Environment		N	Campestanyl and sitosteryl ferulate	Sitostanyl ferulate	Campesteryl ferulate	Total
Years						
Hungary	2005	24	51 ± 6 (53 ± 2%)	32 ± 4 (33 ± 2%)	14 ± 2 (14 ± 2%)	97 ± 11
Hungary	2006	24	54 ± 7 (55 ± 2%)	33 ± 5 (33 ± 3%)	12 ± 2 (12 ± 3%)	100 ± 12
Hungary	2007	24	57 ± 10 (56 ± 2%)	30 ± 5 (29 ± 3%)	15 ± 3 (15 ± 3%)	102 ± 16
Locations						
Hungary	2007	10	56 ± 10 (55 ± 2%)	30 ± 6 (29 ± 3%)	16 ± 4 (16 ± 4%)	101 ±17
France	2007	10	41 ± 5 (52 ± 1%)	25 ± 5 (31 ± 4%)	13 ± 3 (17 ± 4%)	79 ± 10
UK	2007	10	42 ± 5 (52 ± 2%)	23 ±4 (28 ± 4%)	16 ± 4 (20 ± 5%)	81 ± 9
Poland	2007	8	49 ± 5 (52 ± 1%)	31 ± 5 (33 ± 4%)	15 ± 2 (16 ± 3%)	94 ± 10

5.2.3 Relating steryl ferulate contents with wheat kernel characteristics

The correlation between the content of steryl ferulates and thousand kernel weight in bread wheat genotypes was moderately strong and negative, the coefficient of correlation (r) being -0.5562 (**III**; Table 2). The relationship between the total steryl ferulate content and lipid content, on the other hand, was relatively weak and positive (r = 0.3792). Total steryl ferulates also correlated with total phytosterol content (r = 0.5303) and with total stanols (r = 0.7061). No statistically significant correlation was found between the bran yield and steryl ferulate content. The associations of a high content of steryl ferulates with high lipid content and a low thousand kernel weight are also demonstrated in PCA (Figure 14).

5.3 Phytosterols and steryl ferulates in wheat fractions (IV)

5.3.1 Phytosterols in wheat fractions

Total content of phytosterols

The total phytosterol contents of wheat grain and bran fractions produced in three different dry processes varied from 344 to 2117 µg/g. Each process yielded fractions with high phytosterol contents (Figure 18). These fractions included the pearling fraction from the debranning of whole grains, high-purity aleurone 2 separated from the bran and bran fractions from the second (F2A+ and F2B–) and third separation steps (F3B–) of the electrostatic process. The electrostatic separation also gave a minor deposit "loss" fraction with the highest total sterol content. The total contents of the richest fractions were approximately 3– and 6–fold higher compared to the 100% flours and 76% refined flours, respectively, which were the poorest sources of sterols.

Bran fractions produced in the debranning process possessed comparatively high phytosterol contents, whereas the peeling fraction contained 40% less phytosterols than the pearling fraction. The total sterol contents of two aleurone fractions were similar, since the standard aleurone 1 only contained 6% less phytosterols than the high-purity fraction. In the electrostatic separation process, five bran fractions showed higher levels of phytosterols compared with the starting material, whereas the poorest deposit fraction contained about 25% less sterols than the initial bran.

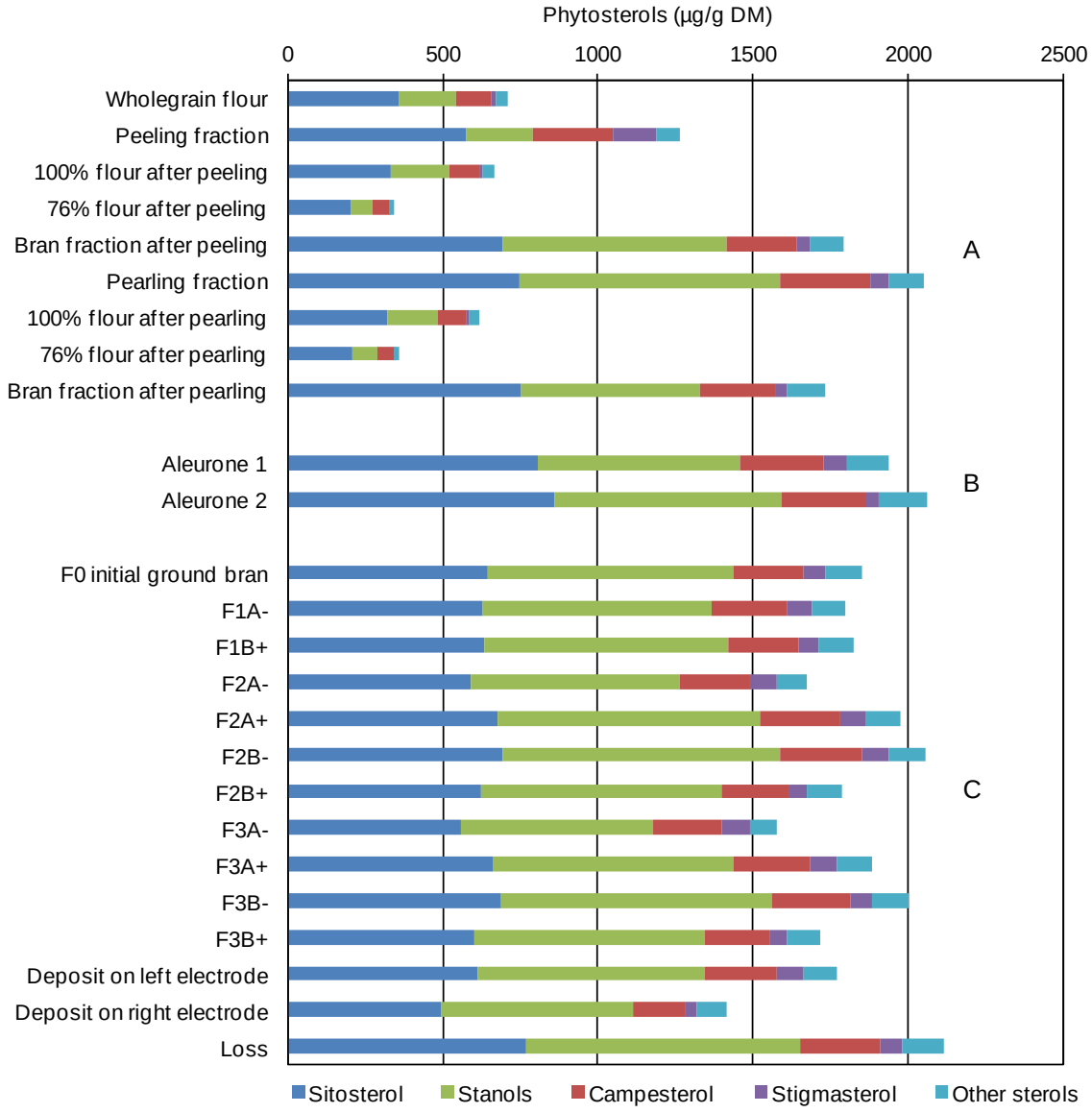

Figure 18. Phytosterol contents (µg/g DM) of the wheat grain fractions produced using a debranning process (**A**) and the wheat bran fractions produced using aleurone separation (**B**) and electrostatic separation (**C**) processes. In this instance, 76% flour corresponds to 76% extraction rate flour.

Phytosterol composition

Sitosterol was the main phytosterol in all wheat fractions and accounted for 34–60% of total sterols (Figure 18). Sitosterol was most predominant in the 76% flours produced after the peeling and pearling processes (See also PCA in **IV**: Figure 3). The lowest relative proportions of sitosterol were found in the pearling fraction and the bran fraction after peeling (debranning process) and in the bran fractions produced in the electrostatic separation process. In such fractions with low proportions of sitosterol, the relative

proportion of stanols (i.e., sitostanol and campestanol) was exceptionally high, at 39–44%. Among other fractions, the stanols comprised 17 to 36% of the total phytosterols. The sterol composition of the peeling fraction differed compared to the other fractions. Stigmasterol and campesterol accounted for 11% and 20% of the total sterols, respectively, in the peeling fraction, whereas in other wheat fractions, 1–6% stigmasterol and 12–16% campesterol were observed. The highest variation in sterol composition was found among the grain fractions produced in the debranning process. The phytosterol compositions of bran fractions processed by electrostatic separation were similar and did not vary considerably from that of the initial bran. The two aleurone fractions separated from wheat bran were also similar in composition.

5.3.2 Steryl ferulates in wheat fractions

Total content of steryl ferulates

Dry processes yielded wheat grain and bran fractions with diverse total steryl ferulate contents, ranging from 6 to 703 µg/g (Figure 19). The highest total steryl ferulate contents were found in the bran fractions F2B- and F2A+ obtained after two separation steps of the electrostatic process. In total, six fractions from the electrostatic separation process showed a higher steryl ferulate content than the initial bran, i.e., over 600 µg/g. A comparable level of steryl ferulates was observed in the bran fraction obtained after peeling, whereas the other fractions from the debranning process had lower steryl ferulate contents. Both aleurone fractions contained approximately 400 µg/g steryl ferulates, which was equal to the pearling fraction, bran fraction after pearling and the deposit fraction with the lowest yield in electrostatic process. Furthermore, the steryl ferulate content of the pearling fraction was 3.5-fold higher compared to the peeling fraction. The most steryl ferulate-rich bran fractions contained up to 10– and 120–fold higher levels of steryl ferulates than the poorest sources, the 100% and 76% flours from the debranning process, respectively.

The steryl ferulates accounted for 1–27% of total phytosterols in the wheat grain and bran fractions (**IV**; Table 3). The highest proportions of sterols esterified with ferulic acid were found in bran fractions obtained from the debranning and electrostatic separation processes, and the lowest proportions were in the 76% flours. In the aleurone fractions, approximately 15% of the phytosterols was bound to ferulic acid.

Steryl ferulate composition

The steryl ferulate compositions of various wheat fractions were similar and did not considerably differ from those found in the wholegrain flour or ground bran used as

starting materials in the processes (Figure 19). Among the electrostatically separated bran fractions steryl ferulate composition varied less than among the grain fractions obtained from the debranning processes. The relative proportions of campestanyl and sitosteryl ferulates (coeluting as a single peak), sitostanyl ferulate and campesteryl ferulate were 52–56%, 30–34% and 12–14%, respectively. Campestanyl ferulate was the main compound accounting for about 45–47 % of the total steryl ferulate content, determined by HPLC-MS.

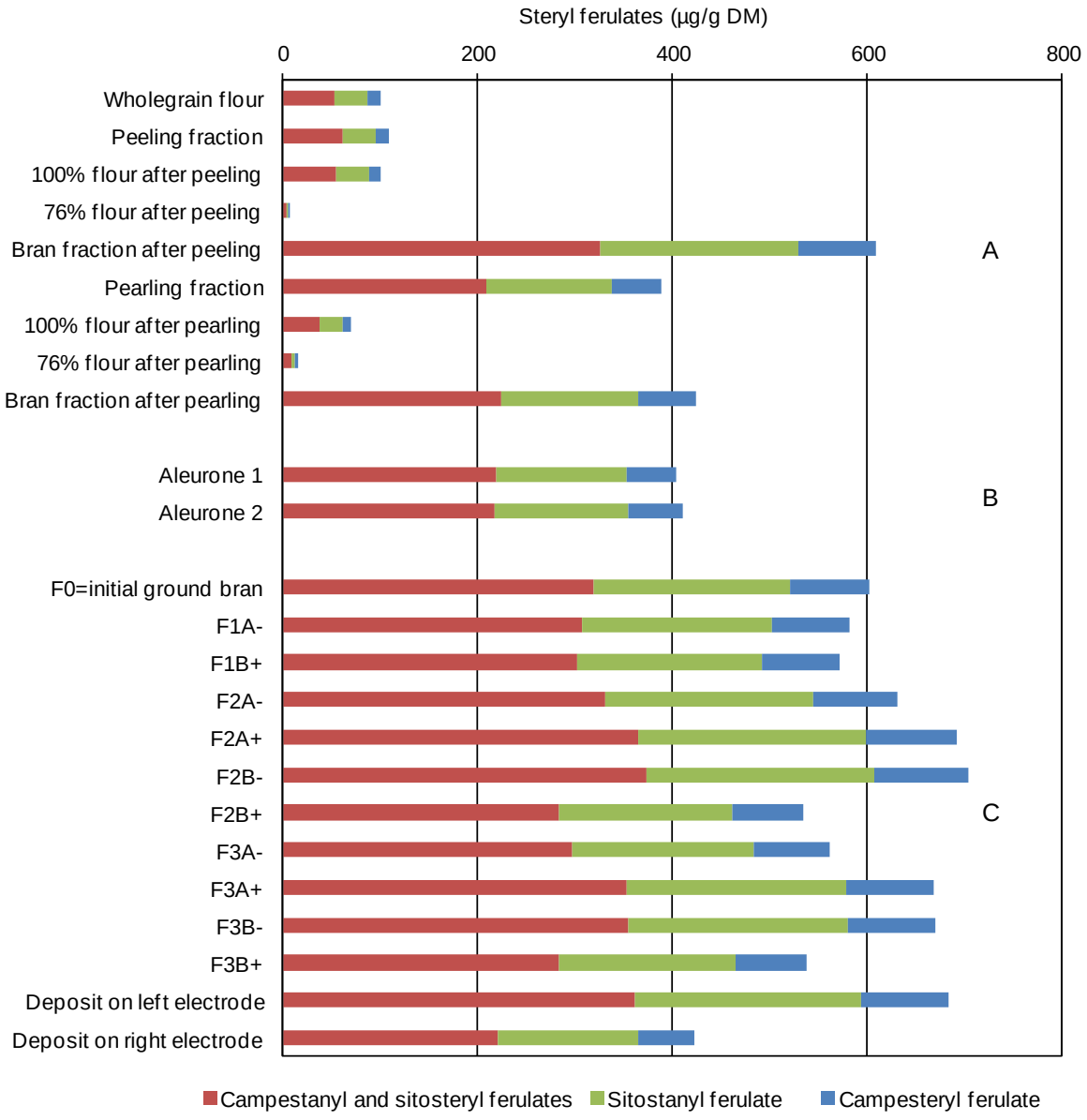

Figure 19. Steryl ferulate contents (µg/g DM) of the wheat grain fractions produced using a debranning process (**A**) and the wheat bran fractions produced using aleurone separation (**B**) and electrostatic separation (**C**) processes. In this instance, 76% flour corresponds to 76% extraction rate flour.

5.3.3 Relating phytosterol compounds with grain tissues

The relationships between the grain tissues and phytosterol compounds and the variation occurring in the composition of the wheat fractions was visualised using PCA (**IV**; Figure 3). High total phytosterol and steryl ferulate contents were associated with high proportions of intermediate layers and aleurone cell content tissues of bran, indicating distribution of the sterol compounds in the inner bran layers of the kernel. Within all 24 wheat grain and bran fractions produced using different dry fractionation methods, the linear correlations between total sterols and these grain tissues were strong and positive, the coefficient of correlation being 0.9109 for the correlation with intermediate layers and 0.8111 for the correlation with aleurone cell content tissue (**IV**; supplementary data, Table A). Steryl ferulates showed a strong positive correlation with the intermediate layers of the grain ($r = 0.9311$). The contents of phytosterol compounds were, on the other hand, negatively associated with the proportion of endosperm tissue. Strong negative correlations were observed between the proportion of starchy endosperm tissue and the total phytosterol content ($r = –0.9235$) or steryl ferulate content ($r = –0.8502$). Total phytosterols and steryl ferulates were also highly related to each other ($r = 0.8786$).

The correlations of individual sterol and steryl ferulate compounds with grain tissues followed, in general, the same pattern as seen for the total contents. However, stigmasterol was strongly associated with outer pericarp tissue, which is the outermost layer of wheat kernel. The correlation coefficient for the correlation of stigmasterol content with the proportion of pericarp tissue was 0.8388. Furthermore, PCA showed that the relative content of sitosterol was negatively related to the total sterol content and bran tissues and positively associated with endosperm tissue. Conversely, the proportion of stanols was positively related to a high total sterol content.

6 DISCUSSION

6.1 Effect of genotype on phytosterol compounds in wheat

6.1.1 Genetic variation in bread wheat genotypes

Phytosterol and steryl ferulate contents

Genetic variation was observed both in the total phytosterol and steryl ferulate contents of bread wheat genotypes (**I**–**III**). During the diversity screen study, all wheat genotypes were grown at the same location during the same season and under similar weather conditions and agronomical practices; therefore, the variation in the total contents of phytosterols (670–959 µg/g DM) and steryl ferulates (79–123 µg/g DM) in the bread wheat lines were likely to arise from genetic factors. Hence, a genotype effect resulted in 1.4- and 1.6-fold differences in the total sterol and steryl ferulate contents of wheat lines, respectively. The variation in the contents of sterol compounds was somewhat lower than in the contents of other bioactive compounds (i.e., folate, tocols and phenolic acids) analysed from the same bread wheat genotypes (Ward et al. 2008; Shewry et al. 2012). Spring wheat types fell in the same ranges with winter types, even though their sowing practices differed; winter wheat genotypes were sown in the autumn, and spring wheat genotypes were planted during the spring. Previously, Alignan et al. (2009) observed that the sowing date did not affect the total sterol content of bread wheat.

Depending on genotype, certain wheat lines tend to develop smaller kernels with higher relative bran and lipid contents than others (**I**–**III**). Since phytosterols are lipid-soluble compounds, and sterol compounds accumulate in the bran (**IV**), genetic factors may cause variation in phytosterol and steryl ferulate contents due to effects on the kernel size. Barron et al. (2011) studied the grain tissue proportions in the selected lines of the diversity screen study, and found that the proportions of both total and individual bran layers varied in wheat kernels depending on the genotype. It was shown in study **IV** that phytosterols and steryl ferulates were unevenly and unequally distributed in the bran layers. The variation in the grain tissue composition may thus result in differences in the sterol and steryl ferulate contents of whole grains with different genetic backgrounds. Furthermore, a variation in lipid content affects the total content of phytosterols because phytosterols accumulate in the lipid-rich germ fraction of the wheat kernel (Nyström et al. 2007); additionally, as suggested in study **IV**, sterols are concentrated in bran layer tissues with high contents of storage lipids (Martelli et al. 2009). Due to the polar ferulic acid moiety, the steryl ferulates are not as lipid-soluble as free or fatty acid esterified sterols and they are only found in low amounts in the germ (Nyström et al. 2007). Therefore, a variation in lipid content does not

have as a strong effect on steryl ferulates as it does on sterols in the present study. As a conclusion, genotypes with a small kernel size that are characterised by high bran and lipid content generally contain high levels of phytosterol compounds.

In addition to the genotype effect, other factors may also cause variation in the contents of sterol compounds. Although the wheat lines were cultivated at the same location to diminish the environmental effects (**I**), it is possible that the soil properties within individual experimental fields in Hungary differed, causing part of the variation occurring in the contents of phytochemicals (Zhao et al. 2009). However, the wheat genotypes were sown in two replicate plots, which should decrease the impact of different soil properties (Rakszegi et al. 2008; Ward et al. 2008). Part of the genotypes originated from Hungary or the same climatic zone as the cultivation site and some adaptation effects may also have occurred.

A large number of bread wheat lines were screened during 2005, and genotypes with high contents of phytosterol compounds could be identified in the present study. The most phytosterol-rich genotypes included winter wheat types Claire, Riband, Atlas 66, Rusalka, Yumai 34, Klein Estrella, Ellvis and CF99105m, as well as the spring wheat types Cadenza and Thatcher. These genotypes contained 929 µg/g or more of phytosterols. Their release years ranged from 1934 to 2002, and six of the genotypes were modern varieties, three were old or transitional varieties, and one was an unregistered germplasm. Phytosterol-rich genotypes originated from all parts of the world; six of the genotypes were of European origin, three were of North and South American origin, and one genotype originated from Asia. The highest levels of steryl ferulates were found in the modern English varieties Claire and Malacca and in the old German variety Disponent, all of which contained over 110 µg/g steryl ferulates. The steryl ferulates were, however, only analysed in selected wheat genotypes, of which a majority originated from European countries. It is noteworthy, that Claire showed both high phytosterol and high steryl ferulate contents. Additionally, phytosterol-rich CF99105 and Atlas 66 were good sources of steryl ferulates.

Bread wheat genotypes with low levels of phytosterol compounds were also observed. The lowest total contents of phytosterols were found in winter wheat genotypes Qualital, Herzog, Sava, Gerek 79, Skorospelka 3B, San Pastore, Blasco, CF99075, Martonvasari 17 and Libellula. These genotypes included four modern, five old or transitional varieties and one germplasm, and were released in 1940-1991. They originated from various parts of Europe. The poorest sources of steryl ferulates were the old winter wheat varieties San Pastore from Italy and Estica from the Netherlands, modern Hungarian winter wheat

variety MV Emese, and spring type germplasm Chinese spring of Chinese origin. San Pastore was thus a poor source of both sterols and steryl ferulates.

The geographical origin of genotypes had little effect on the total contents of phytosterols or steryl ferulates in the present study. The bread wheat genotypes varied widely in their origin; they came from various continents and countries from all over the world (Ward et al. 2008). Possibly, all genotypes did not show their potential expression in the levels of phytosterol compounds when grown in Hungary during the diversity screen study, because they may have adapted to their original growing environment and practices. Landraces, for example, are traditional genotypes that have adjusted to their local environmental conditions and agronomical practices. On the other hand, the varieties originating from Europe may be expected to be better adapted to the European climate and growing locations. However, the genotypes with the highest contents of phytosterols were found to originate from various parts of the world, and those with the lowest contents came from various parts of Europe. A recent study of Andersson et al. (2012) screened the overall biochemical contents of the 129 winter wheat varieties included in the present study. Based on PCA analysis, they suggested that genotypes originating from Eastern Europe and Russia may be low in e.g. sterols, tocols and alkyl resorcinols, whereas those originating from Western Europe may be characterised by high contents of these components.

The year of registration did not have an impact on phytosterol or steryl ferulate contents of genotypes, either. The release years of bread wheat genotypes varied widely, from 1842 to 2004. In addition, a few genotypes were not commercially released. The genotypes with highest and lowest contents of sterols and steryl ferulates included both modern and old varieties. Shewry et al. (2011) reported the same conclusion based on these data, since they found no relationship between the release date and the content of phytosterols or other bioactive compounds in bread wheat genotypes included in the HEALTHGRAIN project. It was concluded that to this day, the selection and plant breeding has not increased or decreased the contents of bioactive compounds.

In the present study, genetic variation of phytosterol and steryl ferulate contents in bread wheat was comprehensively examined in strictly controlled conditions. The 150 bread wheat lines were simultaneously grown at the same location using similar agronomical practices. Prior to this diversity screen study, the effects of genetic factors in wheat phytosterols and steryl ferulates had been studied to lesser extent. The phytosterol contents of two common Finnish wheat cultivars grown at the same area in Finland in 1997 varied slightly, being 763 and 818 µg/g DM (Piironen et al. 2002), which was in accordance to

levels found in this study. In another study, low diversity was observed in the sterol contents of five European winter wheat varieties grown at a single location in Belgium during the same season in 2001–2002 (Ruibal-Mendieta et al. 2004). The phytosterol content varied from 622 µg/g (Rialto) to 655 µg/g FW (Estica), which was lower than in the present study both when comparing the contents of the same cultivars and the extent of variation.

Following the diversity screen study, the effect of genetic factors has been reported in a few more studies. Alignan et al. (2009) found a wide range of genetic variation in the total phytosterol contents of 23 European bread wheat cultivars, grown under organic conditions in France in the 2005–2006 season. The level of phytosterols ranged from 494 to 796 µg/g DM. Four of the cultivars (Apache, Caphorn, Lona and Renan) were also analysed in the present study (**I**), showing higher total phytosterol contents, i.e. 834–896 µg/g. Chen and co-workers (2009) determined the sterol contents of three cultivars grown in 2005 at three locations in Oklahoma, USA, and reported a significant cultivar effect within each location but substantially lower levels of phytosterols (224–403 µg/g DM) than in the present study. The phytosterol contents of five Italian cultivars, grown at the same location in Italy and harvested in 2002, varied from 600 to 677 µg/g DM (Iafelice et al. 2009). One of the cultivars (Mieti) was analysed in the present study, and a higher total content was found (897 µg/g DM) than in their study.

The lower phytosterol values observed in other studies (Ruibal-Mendieta et al. 2004; Alignan et al. 2009; Chen et al. 2009) can be explained by the differences in the analytical methods and the fewer number of sterols analysed. They used direct saponification and excluded the acid hydrolysis step, which has been shown to be a particularly important step in the analysis of sterols from wholegrain wheat flour, releasing the glycosidic conjugates of sterols (Toivo et al. 2001). Iafelice et al. (2009) used both alkaline and acid hydrolyses, but the purification was performed using thin layer chromatography instead of SPE. The differences in the levels of phytosterols may also result from differences in environmental conditions and cultivars analysed. The present study allowed for the comparison of the phytosterol contents in a large number of wheat genotypes without the controversial effects of environmental factors or analytical differences.

Previous knowledge of genetic variation in steryl ferulate contents of wheat is scarce. Seitz (1989) found that the total steryl ferulate contents of seven wheat varieties ranged from 62 to 123 µg/g FW, showing slightly greater diversity than that seen in this study. Later, Hakala et al. (2002) observed relatively low levels and no variation in the total steryl

ferulate contents (62 to 63 µg/g FW) of two wheat grain samples. Other studies have focused on other cereal types, and the effect of genetic factors on steryl ferulates has been reported in rice (Bergman and Xu 2003; Miller and Engel 2006; Heinemann et al. 2008) and corn (Seitz 1989; Singh et al. 2000). The present diversity screen study thus provided valuable new data of the genetic variation on the steryl ferulate contents of wheat.

Phytosterol and steryl ferulate compositions

Genetic factors affected the composition of phytosterols in bread wheat lines (**I-II**). The main phytosterol species in all bread wheat lines was sitosterol, as suggested previously (Piironen et al. 2002; Nyström et al. 2007). Sitosterol comprised approximately half of the total sterols (47–61%), and its content varied 1.6-fold depending on the genotype. Other major 4-desmethylsterols identified were campesterol, sitostanol, campestanol and stigmasterol. The precursors of desmethyl sterols, i.e. 4-monomethyl and 4,4'-dimethylsterols, were present at low concentrations. The greatest variation, up to 2.7–fold differences, was seen in the total content of stanols. Stanols accounted for 11–29% of the total sterols depending on the genotype. The relative content of sitosterol tended to be greater in genotypes with a low total phytosterol content, whereas that of stanols was greater in genotypes with a high total content. This trend may be due to the effect of genetic factors on kernel size and the accumulation of sitosterol in the germ, which is included in wholegrain flour. The germ is known to contain high levels of sitosterols but low levels of stanols. On the other hand, the bran is characterised by a considerably higher content of stanols and a lower content of sitosterols than the germ (Nyström et al. 2007; Plumb et al. 2011). In large wheat kernels, the relative content of the bran layers is low, which may result in a low total content of phytosterols but a high relative content of sitosterol and a low content of stanols concurrently. The present study was the first to demonstrate the genetic variation of phytosterol composition in bread wheat, but other recent studies have also suggested a significant cultivar effect on the sterol profile of wheat (Alignan et al. 2009; Chen et al. 2009; Iafelice et al. 2009).

Ferulic acid conjugates comprised 7 to 9% of the total sterols within bread wheat lines, which is in accordance with previous findings (Hakala et al. 2002; Nyström et al. 2007). The composition of the steryl ferulate fraction differed considerably from that of total sterols. Interestingly, sitosterol and campesterol were the major components of the total phytosterol content (**I**–**II**), whereas the respective stanol species predominated in the steryl ferulates. The most abundant steryl ferulate species was campestanyl ferulate, followed by sitostanyl ferulate, whereas lower levels of unsaturated sitosterol and campesterol conjugates were found (**III**). Similar observations were reported previously (Seitz 1989;

Hakala et al. 2002; Nyström et al. 2007), but the reason for preferential esterification of ferulic acid with saturated phytostanol species is not known. The stanyl ester forms accounted for up to 80% of the total content of steryl ferulates. The minor compounds (three ferulate and two coumarate species) identified in wheat in a recent study of Esche et al. (2012) were not detected in the present study. Trans-campestanyl and sitostanyl coumarates and ferulic acid esters of 24-methylene cycloartanol, Δ^7-campesterol and Δ^7-sitosterol, however, only made up a few percents of the total steryl ferulate content (Esche et al. 2012).

The composition of steryl ferulates was affected by genotype (**III**). The wide ranges observed in contents of campestanyl (including sitosteryl) ferulate (1.6-fold), sitostanyl (1.7-fold) and campesteryl ferulates (2.2-fold) indicate a high variation among genotypes. Previously, the genetic variation of steryl ferulate composition has not been adequately studied. Moderately low variation was found in the distribution of steryl ferulates among seven wheat varieties (Seitz 1989). In another study, the steryl ferulate compositions of two wheat grain samples were similar (Hakala et al. 2002). The wider variation observed in the present study may result from greater number of genotypes studied.

6.1.2 Genetic variation in other wheat genotypes

Phytosterol content

The phytosterol contents were also determined in other wheat types than common bread wheat (**I**). The results showed that primitive einkorn wheat, durum wheat and spelt were better sources of phytosterols than bread wheat and ancient emmer wheat. The differences were up to 1.3-fold when comparing the mean sterol contents of various wheat types and 1.8-fold when comparing single wheat genotypes. The higher phytosterol content in einkorn than in bread wheat is possibly due to the higher total lipid content of einkorn wheat kernels (see Table 9). In the study of Iafelice et al. (2009) slightly higher levels of phytosterols were found in durum, spelt and emmer wheats in comparison to common bread wheat. In contrast to present findings, Ruibal-Mendieta et al. (2004) observed no differences in the sterol contents of spelt and bread wheat. In the present study, the total phytosterol contents of spelt and durum wheat were higher than in previous studies (Ruibal-Mendieta et al. 2004; Ryan et al. 2007; Iafelice et al. 2009), whereas that of emmer wheat was at the same level (Iafelice et al. 2009). Compared to the levels of phytosterols reported in the study of Ruibal-Mendieta et al. (2004), systematically higher levels of phytosterols were observed in the current study, especially in spelt variety Rouquin (573 vs. 845 µg/g FW). These discrepancies most probably arise from a combination of

analytical, genetic and environmental differences. The present study is the first one to publish phytosterol contents in einkorn wheat, since no prior data were available about sterols in this wheat type.

Within other wheat types, the widest genetic variation was observed in the phytosterol contents of durum and einkorn wheat genotypes. The differences within genotypes were, however, only up to 1.3-fold. Slightly wider variation in the phytosterol content of bread wheat is likely to result from the greater number of bread wheat genotypes studied. A somewhat higher diversity was found in the phytosterol contents of spelt and emmer wheat genotypes, when compared to the present study (Ruibal-Mendieta et al. 2004; Iafelice et al. 2009), which likely was because these studies included from 9 to 16 cultivars, and we only analysed five genotypes. On the other hand, Iafelice et al. (2009) observed a lower variation within five durum wheat genotypes than was found in the present study within 10 genotypes.

Phytosterol composition

The same phytosterol species were identified in durum, spelt, einkorn and emmer wheat as in common bread wheat, the most abundant one being sitosterol. The sterol compositions of other wheat types were slightly different from that of bread wheat. Durum wheat contained less sitosterol (45%) compared to bread wheat (52%), which is in accordance with previous findings (Iafelice et al. 2009). Also comparable to their results, slightly higher relative contents of stanols were observed in durum, spelt and emmer wheat than in einkorn and bread wheat. The stanol content of einkorn wheat showed wide variation, ranging up to over 3-fold and being even wider than the variation seen in bread wheat. In other wheat types, the content of stanols varied less, to the same extent as in a previous study (Iafelice et al. 2009). Sitosterol showed less variation in durum, spelt, einkorn and emmer wheat than in bread wheat. Ruibal-Mendieta et al. (2004) found a different sterol profile for spelt than that observed in the present study. In contrast to our findings, they reported a considerably lower relative content for stanols and a higher relative content for sitosterol and campesterol, apparently resulting from analytical differences.

6.1.3 Exploitation of genetic variation

The present diversity screen study (**I**) is hitherto the most comprehensive one to examine the genetic variation occurring in contents and compositions of sterol compounds in bread wheat. It enables the comparison of the phytosterol and steryl ferulate contents in a great number of wheat genotypes without any disruptive environmental factors or analytical

differences. The results have been used in the HEALTHGRAIN project to develop an extensive database of the biochemical composition of wheat. This database allows plant breeders and cultivators to select wheat genotypes with high phytosterol contents or an otherwise favourable phytochemical profile. In theory, such a dataset could also enable the identification of certain wheat genotypes based on their phytochemical compositions.

The selection of genotypes rich in phytosterols and steryl ferulates for food use would lead to an increased intake of these bioactive components and enhanced health. In breeding programs, such genotypes could be used as sources of high levels of bioactive compounds to develop new cultivars with health-promoting benefits. The variation occurring in the content and composition of wheat phytosterols was shown to be highly heritable, approximately 57% in selected wheat lines (Shewry et al. 2012). Such a high heritability indicates that the favorable qualities of various genotypes, such as high phytosterol content, good agronomic properties, good end-use qualities and high yield, can be selected and combined to develop improved, new types of wheat cultivars by classical plant breeding (Shewry et al. 2012).

The composition database has also been used to develop near-infrared (NIR) calibrations for bioactive components and other rapid tools to facilitate the screening of the phytochemical composition in wheat samples, and further, the selection of favourable genotypes (Shewry 2009b; Shewry et al. 2012). Such tools are important for plant breeders because the traditional chemical analyses of phytochemicals usually take time and money. In addition to NIR calibrations, also biochemical and molecular markers and antibody methods are being developed on the basis of the data obtained in the HEALTHGRAIN project (Shewry et al. 2012).

Although genotype was shown to affect phytosterol and steryl ferulate contents, the variation was relatively low, from 1.4 to 1.6-fold. Previous studies with moderately low doses of natural dietary phytosterols have suggested that even small increases in the phytosterol intake may decrease the absorption of cholesterol and reduce serum cholesterol levels (Ostlund et al. 2002; Ostlund et al. 2003; Andersson et al. 2004; Klingberg et al. 2008; Lin et al. 2010). Within British population, for example, a subgroup with an approximately 50 mg higher mean daily intake of phytosterols in a habitual diet resulted in a reduction in LDL cholesterol level, when compared to a subgroup with a lower intake (Andersson et al. 2004). Lowering cholesterol levels decreases the risk of cardiovascular disease, which has positive effects on public health and well-being (Law et al. 1994). As a whole, the health-promoting properties of wholegrain cereals in general should not be

forgotten. Focusing on only a single or few compounds of wheat grains is not sufficient, knowing that wholegrain wheat is a combination of multiple components, such as dietary fibre, vitamins, antioxidants and minerals, which may have various beneficial effects on health. The consumption of wholegrain cereals is known to give protection from several metabolic syndrome-related chronic diseases (Fardet 2010).

One aspect that is worth considering is the impact of the food matrice on the accessibility of phytosterols and steryl ferulates in the gastrointestinal tract. It is possible that the bioaccessibility of sterols in the gut is higher, for example, when they are dispersed in vegetable oils than when they are in more complex matrices, such as cereal foods. According to the scientific opinion of EFSA (2009), the efficacy of phytosterols added to e.g. fat spreads and dairy products is better established than when added to cereals or bread. Thus, the bioaccessibility of sterols and steryl ferulates in the gut from the foods containing wheat needs to be further investigated.

6.2 Effect of environment on phytosterol compounds in wheat

6.2.1 Effect of growing year

Harvesting year did not affect the total contents of phytosterol compounds in bread wheat (**II**–**III**). The genotypes were grown at one location over three consecutive years, and the total sterol and steryl ferulate contents were at the same level from one year to the next. Although the differences among years were not statistically significant, the average phytosterol contents slightly varied by 3%, being highest in 2006, and steryl ferulate contents differed by 5%, being highest in 2007. The lowest levels of sterols and steryl ferulates were observed in 2005.

The wheat lines were grown in controlled experimental fields, and the agronomic practices were similar every year. There were, however, differences in the soil properties of the fields, since the pH and mineral contents varied from year to year (Zhao et al. 2009). The weather conditions also varied slightly among crop seasons (Rakszegi et al. 2008; Shewry et al. 2010a). The first growing season in 2005 was characterised by high precipitation during the harvest, whereas the harvest in 2007 was exceptionally dry. The total precipitation from heading to harvest was, however, was approximately the same every year. The highest average temperature during the plant and grain development stages was observed in 2007. Despite the differences in weather conditions and soil properties among the growing years, significant year-to-year variation was not observed in the contents of sterols and steryl ferulates in bread wheat. The effect of the growing year on phytosterols

or steryl ferulates has not been previously studied in common wheat. The year of growth was shown to have an impact on the contents of phytosterols in rye (Zangenberg et al. 2004) and on the contents of steryl ferulates in rice (Bergman and Xu 2003; Miller and Engel 2006). In the HEALTHGRAIN project, rye cultivars only showed a slight variation in phytosterol contents among years (Shewry et al. 2010b).

Certain individual genotypes showed a higher degree of stability in their phytosterol and steryl ferulate contents among years than others. The low year-to-year variation represents a favourable characteristic for cultivators and plant breeders. The modern German variety Tommi showed the lowest variation in sterol content over the three growing years. Other genotypes with consistent phytosterol contents included the western European varieties Tremie, Estica, Herzog and Claire. In Tremie and Claire, a low variation was combined with a high sterol content (over 900 µg/g). The genotypes with the most stable steryl ferulate content over the growing years included Malacca, Cadenza, MV Emese, Atlas 66 and Herzog. Malacca and Atlas 66 were characterised by both high and stable steryl ferulate contents. On the other hand, Atlas 66 and Cadenza possessed the widest variation among years in their phytosterol contents, along with Avalon, CF99105 and Disponent. The most unstable genotypes in regard to steryl ferulate contents were Valoris, Tremie and Avalon. Thus, the year-to-year stability of the phytosterol compounds in bread wheat genotypes did not have the same tendency; e.g., Tremie had a stable phytosterol content but an unstable steryl ferulate content, whereas Cadenza and Atlas 66 showed stable steryl ferulate contents but unstable phytosterol contents.

Although the year of growth did not significantly affect the total contents of phytosterol compounds, the compositions of individual sterols and steryl ferulates were found to vary slightly among years. The variation in the distributions of these compounds was only moderate, despite being statistically significant. The differences among years in the total contents of sitosterol, campesterol and stanols were only up to 1.1-fold. The total content of sitosterol was highest in 2005 and lowest in 2006, and that of stanols was highest in 2006 and lowest in 2005. Within the steryl ferulate fraction, the campestanyl (including sitosteryl) and sitostanyl ferulates only showed 1.1-fold variations and campesteryl ferulate showed 1.3-fold variation between years. The relative contents of single phytosterol and steryl ferulate compounds only varied by a few percents depending on the year of growth; for example, sitosterol ranged from 49 to 52%, and campestanyl ferulate (including sitosteryl ferulate) varied from 53 to 56%. The effects of growing year on phytosterol or steryl ferulate compositions in bread wheat have not been examined prior to present study.

6.2.2 Effect of growing location

Growing location caused significant variation in the total contents of phytosterols and steryl ferulates within wheat genotypes cultivated at four sites during the same season (**II-III**). Variation caused by the growing location was greater than genetic variation within selected wheat types. Genotypes were grown in different parts of Europe, i.e. Hungary, France, Poland and the UK, representing various climatic conditions. Weather conditions varied widely among the locations in 2007 (Shewry et al. 2010a). During plant and grain development, the lowest mean temperature and highest precipitation were detected in the UK, whereas the Hungarian site was characterised by the highest temperature and considerably lower precipitation. In Poland, the total precipitation was low, being less than a half of the amount observed in the UK. Heading dates varied from May 5th to June 14th, and harvesting dates were from July 5th to August 22nd, being latest in the UK. In addition, soil properties, such as soil type, pH and mineral composition, were different in various locations (Zhao et al. 2009; Shewry et al. 2010a). Agronomic treatments, e.g. the use of nitrogen fertilizer and agrochemicals, also varied among locations. Postharvest conditions were controlled and similar at each location.

Average phytosterol and steryl ferulate contents only varied up to 1.1- and 1.3-fold, respectively, depending on growing location. The highest average contents of sterols and steryl ferulates were observed in bread wheat lines grown in Hungary, where the crop season was hot and dry in 2007. On the other hand, the lowest average contents were found in wheat genotypes grown in the UK (steryl ferulates) and France (sterols and steryl ferulates), which experienced wetter and cooler seasons. Therefore, the weather conditions apparently influenced on the yield of phytosterol compounds. Differences in agronomic conditions may have also caused some of the variation among locations.

High precipitation during plant growth seems to result in a large kernel size, which in turn leads to decreased content of phytosterols and steryl ferulates. This outcome is because in large kernels relative contents of phytochemical-containing lipids and bran are lower than in small kernels. Also, the growing temperature may have an impact on kernel size and thereby also on the contents of phytosterol compounds. Accordingly, elevated temperature and low precipitation may result in higher sterol and steryl ferulate contents. It is also possible that elevated levels of phytochemicals are synthesised in plants in extreme environmental conditions, such as drought or high temperatures, since a protective role of phytosterol compounds against environmental stress has been suggested (Britz et al. 2007). Moderately elevated growth temperature increased the γ-oryzanol content in rice (Britz et al. 2007) and the phytosterol content in soybean seeds (Yamaya et al. 2007). In contrast, a

warm and dry growing season resulted in lower phytosterol contents in rye (Zangenberg et al. 2004). The effects of irrigation on wheat phytosterols was studied by Chen et al. (2009), and their results indicated that irrigation may decrease the total phytosterol content of bread wheat when compared to dryland. This finding is in line with present results, since lower sterol contents were observed in wet growing environments than in dry conditions.

The present observation that the highest phytosterol and steryl ferulate contents were found in Hungarian growing site suggests that some adaptation may have occurred. Wheat genotypes had been cultivated at the same experimental fields in Hungary for two years before cultivation at other locations. Thus, the genotypes may have become adapted to the growing area in Hungary. However, the lack of year-to-year variation in Hungary does not support the occurrence of adaptation. In fact, most of the wheat genotypes included in the trial were genetically homogeneous registered varieties, which are not susceptible to adaptation or natural selection.

This study was the first to comprehensively investigate the effects of the growing environment on bread wheat phytosterols and steryl ferulates, with a large number of genotypes included in the trials. In accordance with present observations, one study by Chen et al. (2009) showed that the location of growth and genetic factors had significant effects on the phytosterol contents of three wheat cultivars, which were grown at three different locations in Oklahoma, USA, in 2005. Other studies have focused on other cereal types. Määttä et al. (1999) found no variation caused by growing location in the sterol contents of seven oat cultivars when cultivated at three locations in Sweden in 1996. A low environmental variation was observed in the phytosterol contents of five rye genotypes included in the HEALTHGRAIN project (Shewry et al. 2010b). On the other hand, the total phytosterol contents of two soybean varieties varied depending on the growing location in Japan (Yamaya et al. 2007). The location of growth also had a significant effect on steryl ferulate content in seven rice cultivars, which were cultivated at four locations in the USA from 1999 to 2000 (Bergman and Xu 2003), thirty brown rice cultivars, which were cultivated at various sites and seasons in southern Europe (Miller and Engel 2006) and six corn hybrids, which were cultivated at various locations in the USA (Singh et al. 2000). Therefore, the variation in cereal phytosterols and steryl ferulates by growing location that was observed in the present study is supported by previous findings.

The extent of variation in phytosterol and steryl ferulate contents among locations differed depending on genotype, and wheat varieties with stable phytochemical contents were identified. Winter wheat genotypes MV Emese, Gloria, Obriy and Atlas 66 had stable

phytosterol contents over the growing locations. Atlas 66 possessed both high and stable total sterol content. Steryl ferulate contents were moderately stable in genotypes Estica, Malacca and MV Emese. Malacca was also characterised by a high steryl ferulate content and high year-to-year stability, thus being a promising cultivar for plant breeding and cultivation purposes and further for health-promoting applications. MV Emese showed low variation among locations in both sterol and steryl ferulate contents and also low year-to-year variation of steryl ferulates. The widest variation over growing locations was found in the phytosterol content of winter wheat genotypes Riband, Disponent and Lynx. The winter wheat types Tremie and Valoris and the spring type Chinese Spring showed high variation in total steryl ferulate content among locations. Tremie and Valoris also had wide year-to-year variation.

Phytosterol and steryl ferulate compositions varied only moderately depending on growing location. The mean contents of sitosterol, campesterol and stanols ranged 1.1-, 1.2- and 1.4-fold, respectively. Thus, the variation of stanol content may cause more differences in the total content of phytosterols than that of other main sterol components. The highest mean contents of sitosterol and campesterol were observed when genotypes were grown in the UK. In contrast, the stanol content was high in genotypes cultivated in Hungary and low in those grown in the UK. Again, a high relative content of sitosterol was accompanied by a low total phytosterol content, and a high relative content of stanols occurred with a high total phytosterol content. Chen et al. (2009) has previously reported on the variation in phytosterol compositions of three bread wheat cultivars, which were grown at three different locations during the same year. The contents of the main steryl ferulate species, campestanyl (including sitosteryl) and sitostanyl ferulates exhibited 1.4-fold differences depending on growing location, whereas the content of campesteryl ferulate varied slightly less. As expected, the genotypes grown in Hungary had high contents of campestanyl and sitostanyl ferulates. The variation in steryl ferulate composition due to the growing location has not been studied before but has been demonstrated in other cereal types. Distribution of steryl ferulates varied in rice depending on growth location (Miller and Engel 2006) and temperature (Britz et al. 2007).

6.2.3 Overall variation

Genetic factors had a significant effect on phytosterols and steryl ferulates in wheat, resulting in up to 1.4- and 1.6-fold differences, respectively. Within selected genotypes, the effect of environment was greater than the effect of genotype. The growing location caused significant variation, whereas the growth year did not affect the contents of sterol compounds in present experimental conditions. Among all environments, the highest mean

phytosterol and steryl ferulate contents were found in bread wheats grown in Hungary in 2006 and 2007, respectively, and the lowest were seen in those grown in France in 2007. This variation was 1.2-fold for sterols and 1.3-fold for steryl ferulates.

When comparing single genotypes, the highest total phytosterol content was observed in spring wheat Cadenza grown in Hungary in 2006 (1039 µg/g DM) and the lowest in winter wheat Herzog grown in France in 2007 (645 µg/g), showing a 1.6-fold difference. The highest average sterol content and good stability within various environments was detected in winter type Claire. Chinese Spring, Gloria, Isengrain and Rialto had moderately high and stable phytosterol contents over the different years and locations. The highest steryl ferulate content was in winter wheat type Tremie when grown in Hungary in 2007 (126 µg/g DM), and the lowest was in Cadenza grown in France in 2007 (66 µg/g DM). The difference between these contents was 1.9-fold. The genotype with the highest average steryl ferulate content was Crousty. Malacca had a combined high total steryl ferulate content and good year-to-year and site-to-site stability. However, fewer genotypes were analysed for steryl ferulates than for sterols.

The wheat genotypes included in the present study have been scored on the basis of their overall phytochemical contents (Ward et al. 2008; Shewry et al. 2011). According to Shewry et al. (2011), wheat lines with high scores for both phytochemicals (tocols, sterols, alkylresorcinols, phenolics and folates) and dietary fibre components included Lynx, Campari and Moulin. A diversity screen trial showed that CF99105, Disponent, Hereward and Moulin were genotypes with high phytochemical and fibre contents (Ward et al. 2008).

6.2.4 Exploitation of environmental variation

The environmental effects on sterols and steryl ferulates have not been studied before in wheat as thoroughly as in present studies (**II**-**III**). The data obtained allows the selection of genotypes showing high stability in their phytochemical content over the growing years and locations. Such stable genotypes are suitable for cultivation in various environmental conditions and in a range of climatic zones, ensuring that the composition of bioactive components remains unchanged regardless of the growing environment. This dataset also enables the selection of genotypes, which are favourable for cultivation in certain climatic conditions and regions.

The environmental variation in phytosterols and steryl ferulates of wholegrain wheat was not as broad as in several other wheat phytochemicals (e.g. tocols, folate and phenolic acids) that were determined during the HEALTHGRAIN project (Shewry et al. 2010a).

However, even small changes in the intake of phytosterols may have a positive impact on health (Andersson et al. 2004).

In general, the natural variation in wheat phytosterols and steryl ferulates caused by environment and genetic factors (**I**-**III**) was lower than that observed in wheat fractions (**IV**). To increase the intake of phytochemicals, one feasible method would be to exploit wheat fractions more efficiently. The utilisation of cereal types containing more phytosterols than wheat (such as rye) should also be taken into consideration.

6.3 Effect of dry fractionation on phytosterol compounds in wheat

6.3.1 Variation in wheat fractions

Phytosterol and steryl ferulate contents

Wheat was fractionated into various flour and bran fractions using three dry processes, i.e. conventional debranning, aleurone separation and a novel electrostatic separation technique. Each dry process yielded wheat fractions with high contents of phytosterols and their ferulate conjugates. The best sources of phytosterols were the pearling fraction, high-purity aleurone and bran fraction F2B- obtained using electrostatic separation. The highest steryl ferulate levels were found in the bran fraction after peeling and in bran fractions such as F2B- and F2A+ from electrostatic separation. The flour fractions obtained in the debranning process were the poorest sources of phytosterols and steryl ferulates, with the 100% flours having higher contents of these compounds than the refined flours. The minor loss fraction obtained in electrostatic separation showed an exceptionally high sterol content but is not useful in practice. The loss fraction was collected from the tribo-charging line and had a low yield (Hemery et al. 2011).

The fractionation resulted in high variation in the phytosterol contents, since the total phytosterol contents exhibited a 6-fold range (from 344 to 2117 µg/g DM) and steryl ferulate contents a 120-fold range (from 6 to 703 µg/g). The wide ranges can be explained by differences in the amount of bran tissues in the samples, assuming that the sterols and steryl ferulates are concentrated in the bran (Hakala et al. 2002; Nyström et al. 2007). Consequently, pure bran fractions possessed the highest contents, and refined flours, being almost absent of bran tissues, possessed the lowest contents. The wider variation in steryl ferulate contents compared to sterol contents of the wheat fractions is explained by differences in the distribution of the compounds in the wheat kernel. Phytosterols are present in all plant cells, including the endosperm cells, since they are located in the cell

membranes that regulate membrane fluidity and permeability (Schaller 2003). Steryl ferulate conjugates, instead, are almost exclusively found in the outer layers of the kernel (**IV**), possibly because their function in the plant cells may differ from that of the free sterols or other sterol conjugates. Steryl ferulate may protect grains from microbial activity, oxidative damage and other environmental stress (Seitz 1989; Britz et al. 2007; Islam et al. 2009). However, other possible functions of steryl ferulates in plants are still unknown. The greater accumulation of steryl ferulates in the bran compared to sterols inevitably results in a wider variation in wheat fractions.

The present results are in line with previous publications that reported higher levels of phytosterols and steryl ferulates in the bran than in flours (Hakala et al. 2002; Nyström et al. 2007). The total contents of phytosterols and steryl ferulates in wheat bran, wholegrain flours and refined flours found in the present study are comparable to previous experimental results (Seitz 1989; Collins et al. 2002; Hakala et al. 2002; Normén et al. 2002; Piironen et al. 2002; Nyström et al. 2007; Kamal-Eldin et al. 2009; Esche et al. 2012). More specific fractions of bran have not been extensively studied. The aleurone fractions produced using a similar separation process as the one in the present study contained slightly higher levels of phytosterols (2190 µg/g) and lower levels of steryl ferulates (260 µg/g) than in the present study (Buri et al. 2004a). The small differences compared to our values probably result from differences in the type of starting material and the purities of the aleurone fractions. The variation occurring in the contents of phytosterol compounds within different types of fractions may arise from differences in the starting materials, since the genetic factors are known to affect the levels of phytosterols (**I**) and steryl ferulates (**III**) and the proportions of wheat grain tissues (Barron et al. 2011) in wholegrain wheat. Hence, genotypes may develop wheat kernels with differing size and bran content, and also with differing distribution of bran layers, thus causing variation in the contents of sterol compounds. Additionally, the type of processing technique and method used for analysis of sterols may affect the levels of these compounds (Toivo et al. 2001). In the present study the various wheat grain and bran fractions were produced from the same starting material, a single wheat genotype that was grown at one location in 2006. The wheat kernels were fractionated into 24 distinct portions using three different processes. Thus, the present study is the most extensive one to comprehensively analyse the variation of phytosterol compounds in grain and bran layers. Phytosterols are also known to accumulate in the germ fraction of the wheat kernel, and higher contents of sterols have been observed in the wheat germ than in the bran (Piironen et al. 2002; Nyström et al. 2007). The steryl ferulate content of the germ is negligible.

Phytosterol and steryl ferulate compositions

The predominant sterol and steryl ferulate species in all bran and flour fractions were sitosterol and campestanyl ferulate, respectively, similar to those in whole grains (**I**–**III)** and previous findings (Hakala et al. 2002; Nyström et al. 2007). The dry fractionation processes yielded wheat fractions with varying phytosterol compositions, whereas the composition of steryl ferulates showed less variation, being similar in flour and bran fractions in general. The variation in relative contents of sitosterol, campesterol, stanols and stigmasterol in bran and flour was broad: 34–60, 12–20, 17–44 and 1–11%, respectively. Most of this variation occurred in wheat grain fractions produced in debranning process, whereas in fractions from other processes, the sterol composition was more stable. The total contents of sitosterol and campesterol varied 4- and 5-fold, respectively, depending on the fraction, whereas stanols and stigmasterol showed more extensive variation with 13- and 51-fold differences. Sitosterol was the predominant sterol in flour fractions, while the bran-containing fractions had higher relative contents of stanols than flours. This finding is possibly because the germ (included in the flour fraction) is especially rich in sitosterol and poor in stanols, while high contents of stanols are found in the bran (Nyström et al. 2007). The proportion of sitosterol was high, and that of stanols low in fractions with low total content of phytosterols.

Previous studies reported similar phytosterol compositions in wheat bran (Nyström et al. 2007; Kamal-Eldin et al. 2009) and whole grains (Piironen et al. 2002; Nyström et al. 2007; Iafelice et al. 2009) as in the present study, in general, and also a higher relative content of sitosterol in wholegrain wheat in comparison to wheat bran. However, in a few studies, slightly higher levels of sitosterol and lower levels of stanols were observed in wholegrain wheat (Normén et al. 2002), refined flour (Piironen et al. 2002) and bran (Normén et al. 2002; Piironen et al. 2002) when compared to this study, probably because of differences in the sample materials caused by genetic and environmental factors. The phytosterol composition of a standard aleurone preparation studied by Buri et al. (2004a) is in accordance with present findings. In this same study, steryl ferulate compositions of standard and high-purity aleurone were determined, and only slight differences in compositions (with a lower content of the main compound and a higher level of sitostanyl ferulate) were found compared to the present study. Such differences were possibly due to the use of different wheat varieties as a starting material in the aleurone separation processes.

The degree of phytosterol ferulation varied considerably (27-fold) in different fractions. In bran fractions, a higher relative content of phytosterols was ferulated than in the flour

fractions. The greater esterification of phytosterols with ferulic acid in the outer parts of the grain is probably related to the accumulation of ferulic acid in the bran (Barron et al. 2007). In this study, a slightly higher degree of ferulation was observed in wholegrain flours (or 100% flours) and bran fractions than in previous ones (Hakala et al. 2002; Nyström et al. 2007). The relative contents of campesteryl (including sitosteryl), sitostanyl and campesteryl ferulates did not considerably vary among wheat fractions; thus, the overall composition was quite stable from fraction to fraction. However, the contents of individual steryl ferulate components were remarkably diverse, ranging up to 128-fold, as did the total content. Steryl ferulate compositions in wholegrain wheat and bran were similar to those reported earlier (Seitz 1989; Collins et al. 2002; Hakala et al. 2002; Nyström et al. 2007; Esche et al. 2012). As an exception, a somewhat higher relative content of campesteryl ferulate was observed in a few previous studies (Collins et al. 2002; Hakala et al. 2002) when compared to this study.

6.3.2 Distribution in the wheat kernel

The results of study **IV** clearly show the accumulation of phytosterols and steryl ferulates in the bran layers of the wheat grain. The fractions rich in bran tissues (e.g. aleurone fractions) contained considerably higher levels of sterol compounds than those rich in starchy endosperm tissue (e.g. flour fractions). The accumulation of these compounds in the outer layers of the grain has been previously suggested; phytosterol and steryl ferulate contents of wheat bran were found to be significantly higher compared to wholegrain flours (Hakala et al. 2002; Nyström et al. 2007). Furthermore, many other bioactive compounds (such as dietary fibre, phenolic acids and alkylresorcinol) are also known to be concentrated in the bran layers or germ instead of the inner endosperm-rich part of the grain (Adom et al. 2005; Landberg et al. 2008; Fardet 2010).

Phytosterols and steryl ferulates were distributed unevenly and unequally within the bran layers. The compounds were more concentrated in the inner bran layers than in the outermost pericarp tissue. This trend was indicated by both PCA (**IV**; Figure 3) and the lower sterol and steryl ferulate contents of the peeling fraction compared to the bran fractions. The pearling process, on the other hand, resulted in a 30% decrease in the steryl ferulate content of the bran but only a 3% decrease in the total sterol content (bran after peeling vs. bran after pearling), suggesting that the compounds were not similarly distributed on the bran layers. The different localisation was further confirmed by correlation studies. Phytosterols accumulated in both intermediate layers and intracellular contents of the aleurone layer, whereas steryl ferulates were more prevalent in the intermediate layers. It is obvious that these compounds are not highly localised in a single

bran layer (as is the case with e.g. alkylresorcinols, which are highly localised in the testa) but instead are concentrated in certain areas. Phytosterols are present in all grain tissues, since they are structural and functional components of plant cell membranes.

Within bran layers, the accumulation of phytosterols in the intracellular contents of aleurone cells and not in the aleurone cell walls is evident because sterols are found in the intracellular membranes of plant cells (Tjellström et al. 2010; Cacas et al. 2012). Aleurone cells are a generative part of wheat grain, and phytosterols apparently have role in cell proliferation and other important cell functions needed for plant growth (Piironen et al. 2000). In addition, the storage lipids of wheat bran occur predominantly within aleurone cells, more precisely in aleurone granules and also in three cuticles within the intermediate layers and the outer pericarp (Martelli et al. 2009); lipid soluble phytosterols are probably localised with storage lipids. Phytosterols and steryl ferulates have been previously detected in aleurone-containing wheat preparations (Buri et al. 2004a).

Within the three intermediate layers (the hyaline, testa and inner pericarp layers), phytosterols and steryl ferulates are possibly more concentrated in the hyaline layer and the testa than in the inner pericarp, which contains degenerated empty cells (Morrison 1976). Furthermore, the hyaline layer is rich in ferulic acid (Barron et al. 2007), which supports the synthesisation and accumulation of steryl ferulates in hyaline tissue. A previous study (Hemery et al. 2011) showed a strong correlation between the contents of steryl ferulates and alkylresorcinols in wheat, suggesting that steryl ferulates may be localised close to the alkylresorcinols in the testa (Landberg et al. 2008). Sterols were also observed in the testa of barley kernels (Briggs 1974).

Seitz et al. (1989) previously detected steryl ferulates in wheat fractions composed of inner pericarp and aleurone, but did not observe them in the outer pericarp layer. In the present study, however, the outer pericarp tissue contained both sterols and steryl ferulates. In wheat kernels, the outer pericarp consists of empty cells with thick cell walls formed by cytoplasm degeneration (Morrison 1976; Anson et al. 2012), which in turn may explain the relatively low total contents of phytosterol compounds in outer pericarp tissue. On the other hand, the cell membranes (containing phytosterols) were found to persist to the final stages of degeneration in inner pericarp cells (Morrison 1976), and the outer pericarp also contains a lipid-rich cuticle (possibly containing phytosterol compounds) as an outermost layer (Martelli et al. 2009).

The distribution of phytosterol compounds in wheat kernels and bran tissues was not adequately studied prior to the present study. In a previously published wheat grain tissue correlation study (Hemery et al. 2011), the distributions of numerous phytochemicals in the wheat kernel were traced, but the results concerning sterols and steryl ferulates were based on the same experimental data as discussed in the present study. The determination of phytosterol compounds in hand-dissected, single botanical layers of a wheat kernel would give further knowledge of the distribution of these phytochemicals. Dry fractionation or other technological processes can not produce such precise fractions with only one tissue present.

The composition of phytosterols differed in the inner and outer layers of the wheat kernel. Endosperm tissue was rich in sitosterol, and stanols were more abundant in the bran tissues. No variation was found in the steryl ferulate compositions within the wheat kernel or the various bran layers, although the total content of steryl ferulates varied in different parts of the kernel. Phytosterols, however, showed some differences within the bran layers. Stigmasterol was found to accumulate in the outer pericarp tissue. In other plant species, such as tomato, accumulation of stigmasterol on the outer layer of fruit has been suggested to relate to ripening, aging, cell disruption and cell senescence (Whitaker 1988; Stalleart and Geuns 1994; Moreau et al. 2002). Stigmasterol was shown to regulate plant membrane functions less effectively than e.g. sitosterol, despite only a slight difference in the chemical structures of these compounds (Schuler et al. 1991; Marsan et al. 1996). In potato tubers, the increase in stigmasterol content during aging was suggested to result in membrane rigidification and increased membrane permeability (Zabrouskov and Knowles 2002), because of the disordering effect of stigmasterol in phospholipid bilayers. Increased expression of certain gene encoding sterol C-22 desaturase (which converts sitosterol to stigmaterol) was responsible for stigmasterol accumulation in barley and tomato fruit during ripening (Whitaker and Gapper 2008; Tang et al. 2011). The barley chromosome addition lines of common wheat also showed elevated stigmasterol levels in seedlings but not in the seeds themselves. In addition, the incorporation of pathogenic microbes was shown to induce the conversion of sitosterol to stigmasterol through stimulation of sterol C-22 desaturation in *Arabidopsis thaliana* leaves, possibly enhancing the plant resistance to bacterial leaf infection (Griebel and Zeier 2010). Although this phenomenon has not been studied in wheat grains, it is possible that stigmasterol may be concentrated in the ruptured cells of the outer pericarp during maturation.

6.3.3 Exploitation of variation in wheat fractions

Wheat fractions

The wide range of wheat grain and bran fractions produced using various dry processes enabled us to study the variation of phytosterol components within the wheat kernel. Knowledge of the distribution of these phytochemicals in the bran layers of the wheat kernel can be utilised to produce sterol and steryl ferulate-rich grain fractions. Such fractions can possibly be exploited in cereal foods to enhance the intake of health-promoting components in a natural diet. The fractions with high levels of phytosterol compounds could also be used as starting materials for extraction of phytochemical preparations. A total lipid extract of wheat bran was previously shown to contain high levels of phytosterols, over 17 000 µg/g (Jiang and Wang 2005).

Bran is a by-product of the conventional milling process and is mainly utilised as a feed for animals. At the same time, phytosterols and other potentially valuable components are are underutilised when they are used as low value rations for animal feeds. Unfortunately, for the most part, only refined phytochemical-poor endosperm flour is used by humans. Thus, it seems quite feasible and sustainable to start exploiting these discarded bran fractions as food ingredients. However, as discussed in a previous section (6.1.3), the bioaccessibility of sterols and steryl ferulates from wheat grains and bran fractions remains to be understood adequately enough to ensure if (and to what extent) these components are available in the small intestine for their bioactive function. In addition to phytosterol compounds, wheat bran contains numerous other bioactive components, such as phenolic acids, alkylresorcinols, betaine, choline, folate and minerals (Anson et al. 2012). Various wheat processing techniques, such as germination, debranning, milling, bran fractionation, bread making, fermentation and enzymatic treatments, have been shown to affect the contents and bioaccessibilities of bioactive cereal compounds in the final products (Hemery et al. 2010; Anson et al. 2012), suggesting that the bioaccessibility of phytosterols from the cereal food matrix could possibly be enhanced by processing.

The suitability of the bran fractions for food use needs to be established. The addition of bran fractions into foods, such as bread, affects the sensory and functional properties of the products. The aleurone fractions may potentially be used in food applications, since aleurone preparations have a pleasant taste, colour and texture as ingredients in cereal foods (Buri et al. 2004b; Brouns et al. 2012). On the other hand, the addition of aleurone or bran fractions in the dough was shown to decrease the volume and gluten yield of the wheat bread (Noort et al. 2010). The reduction in the size of aleurone and bran particles

resulted in increased negative effect. “Healthflour” is a mixture of endosperm flour and pearling fraction, which has been developed during the HEALTHGRAIN project and is suggested not to have adverse effects on technological quality (Delcour et al. 2012). It has also been shown that the conventional wheat milling process yields milling fractions, which have high phytosterol contents comparable to wheat bran but an otherwise flour-like appearance and other characteristics, e.g., ash and fibre contents (Nyström et al. 2007).

In many Western countries, the consumers favour wheat products made of refined flours over the wholegrain- or bran-containing foods, which differ in colour, flavour and taste from the refined ones. The present results show that conversion to the consumption of wholegrain products would considerably increase the intake of health-promoting phytosterol compounds. Thus, new technologies are needed to develop cereal foods and preparations, which contain high levels of bioactive constituents of wholegrain wheat or wheat bran but are, nevertheless, acceptable for consumers. Such technologies have been under development during the HEALTHGRAIN project (Delcour et al. 2012). One of the inventions has been Healthflour, discussed above, which is rich in bioactive compounds but contains low levels of grain components (such as the contaminants of the outer pericarp tissue) that would have negative effects on quality and safety of the product. In addition, the consumer awareness of the beneficial properties of wholegrain and bran-containing cereal foods should be increased to enhance the attitudes towards healthier cereal foods.

Dry fractionation processes

Debranning is an industrial-scale dry fractionation process, which removes the bran layers of the grain gradually using peeling and pearling prior to milling. During the peeling process, approximately 3.5% of the outer grain layers are peeled off by rubbing the grains against each other in a peeler machine (Eugster and Gerschwiler 2006; Hemery et al. 2009). The peeling fraction obtained in the process is mostly composed of the outermost pericarp tissue, which may contain microbial contaminants, heavy metals and dust. The bacteria and moulds distributed in the pericarp of the wheat kernel can be effectively removed using debranning (Laca et al. 2006). Debranning of the outhermost kernel layers before milling may also improve the yield and refinement of wheat flour (Dexter and Wood 1996). Since the peeling does not considerably decrease the level of the phytosterol compounds in the wholegrain flour, peeling of the grains prior to milling to remove contaminants from the outermost grain layers does not significantly affect the phytosterol intake from the wholegrain products. The peeling process did not decrease the steryl ferulate content of bran, and the bran separated after the peeling was the most steryl ferulate-rich fraction obtained in the debranning processes. During the pearling process

(following the peeling) the grains were rubbed against an abrasive stone to remove 3% more of the outer layers (Hemery et al. 2009). The pearling fraction obtained in the process was the best source of phytosterols within the debranning fractions and a good source of steryl ferulates as well. Previously, pearling was used to produce phytosterol-rich fractions from barley and rye grains (Lampi et al. 2004; Liu and Moreau 2008). After the debranning processes, the peeled and pearled grains were milled to 100% and 76% flours. The 100% flours were better sources of phytosterols and their ferulic acid conjugates than the refined flours due to their higher content of bran tissues, since the sterol compounds are concentrated in the bran (**IV**).

The fractionation of wheat bran using an aleurone separation process included two steps. First, the bran was ground, air-classified and sieved to give the standard purity aleurone preparation, which was then further purified to a higher purity aleurone using electrostatic separation (Hemery et al. 2009). The total contents and compositions of the phytosterol compounds were quite similar in both isolated wheat aleurone fractions, regardless of the purity level. Buri et al. (2004a) previously found elevated levels of phytosterols and steryl ferulates in aleurone preparations and also showed the similar steryl ferulate compositions of the standard and high-purity aleurones. During the second purification step, the proportion of aleurone material in the fraction increased from 65 to 79%, but the sterol content was only increased by 6% and the steryl ferulate content by 1%. Thus, the first separation step of wheat bran was adequate to obtain phytosterol-rich fractions. Both aleurone fractions contained high levels of phytosterols and moderately high levels of steryl ferulates, being comparable to those in the pearling fraction from the debranning process.

The third dry fractionation technique used for the production of wheat bran fractions for study **IV** was a novel electrostatic separation process (Hemery et al. 2011). Electrostatic separation yielded new types of bran fractions, dividing bran particles into several specific fractions based on their chemical composition. The phytosterol and steryl ferulate contents of wheat bran could be slightly elevated using electrostatic separation. The total content of phytosterols was enriched by up to 11% and that of steryl ferulates up to 17%. The second step of the separation process yielded higher phytochemical levels than the third step, indicating that two electrostatic separation steps were sufficient to produce bran fractions rich in phytosterol compounds. Using, for example, a pearling fraction as a starting material in the electrostatic separation could possibly result in fractions with even higher phytosterol contents than were found in the present study.

Debranning, aleurone separation and electrostatic separation could be used for production of wheat grain or bran fractions with high contents of phytosterols and their ferulate conjugates. When producing wheat fractions using other processes than conventional milling, the energy consumption and the costs are greater (Hemery et al. 2007). Thus, the economic feasibility of the dry fractionation processes needs to be estimated, to determine if the production of fractions rich in bioactive compounds is economically viable using these processes.

7 CONCLUSIONS

Cereals are an important natural source of phytosterols and their ferulic acid esters. In this thesis, natural variation occurring in wheat phytosterols and steryl ferulates were studied, with a particular focus on the effects of genotype, environment (growing year and location) and dry processing on sterol compounds in bread wheat. The present study is hitherto the most extensive one published in this field. The data obtained have already been exploited by establishing a database, which can be utilised by plant breeders and growers to review the phytochemical profiles of various cereal genotypes and to select genotypes with high and stable contents of bioactive grain components.

The contents of phytosterols and their ferulic acid conjugates vary depending on genotype; wheat genotypes with high levels of sterol compounds were identified. The origin or release date of the genotypes does not have a considerable effect on sterol and steryl ferulate contents. The wheat type may have such an effect, since ancient einkorn wheat and durum wheat were slightly richer in phytosterols than common bread wheat. Phytosterol and steryl ferulate profiles of various genotypes show considerable genetic variation as well. Sitosterol is the most abundant phytosterol, whereas stanols are the major species in the steryl ferulate fraction. The highest variation was found in stanol contents. The genotypes with high total contents of sterols are characterised by a high relative content of stanols and a low relative content of sitosterol.

Environmental factors also have an influence on the contents and compositions of phytosterol compounds. The highest contents were observed in genotypes cultivated in Hungary during a dry and warm season and the lowest contents in those cultivated in the UK and France under cold and wet growing conditions. Differences in both climate and soil properties possibly contributed to the variation observed in wheat phytosterols and steryl ferulates among the growing locations. Within the bread wheat lines grown at various locations, the effect of growing location on phytosterol and steryl ferulate contents was greater than the effect of genotype. The year of growth, on the other hand, does not have an effect on total sterol or steryl ferulate contents of bread wheat genotypes. Some genotypes show wider environmental variation than others; based on present data, several genotypes with stable contents of phytosterol compounds over the growing years and locations were identified.

Small wheat kernels were shown to be characteristic of certain genotypes and were also caused by some environments with low precipitation and high temperature. Small kernels

possess high relative bran and lipid contents and therefore show higher levels of phytosterols and steryl ferulates than the large kernels, which in turn may cause some of the natural variation seen in contents of sterol compounds.

Both novel and conventional dry fractionation processes yielded wheat grain and bran fractions with high total sterol and steryl ferulate contents. The sterol compositions of the fractions and grain tissues vary considerably, whereas the steryl ferulate composition is similar in various fractions and in all parts of the wheat kernel. Phytosterols and steryl ferulates are concentrated in the bran layers of the wheat kernel and are unevenly distributed within the bran layers; sterols accumulate in intracellular aleurone tissue and the intermediate layers, and steryl ferulates accumulate in the intermediate layers. Further distribution within the intermediate layers still needs to be studied in more detail, although the testa and hyaline layers apparently contain the highest levels of phytosterol compounds. Additionally, the accumulation of stigmasterol in the outermost pericarp tissue of the wheat kernel remains unexplained.

These findings support the use of wholegrain and bran-containing products instead of cereal foods prepared from refined flours. Whole wheat grains and fractions with high phytosterol contents could be introduced into cereal foods to enrich the phytochemical content and to enhance the intake of these health-promoting compounds. Phytosterols have been shown to significantly lower serum cholesterol levels, which may reduce the risk of cardiovascular disease. Since health benefits have also been observed with relatively low doses of naturally occurring phytosterols in the normal daily diet and not only with fortified functional food products, the natural enrichment of cereal foods with sterols and steryl ferulates may promote public well-being and improve health. Even modest changes in the dietary intake of phytosterol components may result in considerable improvements on health over a lifetime.

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9 APPENDIX

Supplementary table 1. Release years, countries of origin and types of the wheat lines. Selected bread wheat genotypes grown in various environments during 2005–2007 are marked with asterisk (*).

N	Genotype	Release year	Country of origin	Type
1	Agron	1980	Germany	winter
2	Agrounia	1987	Serbia	winter
3	Akteur	2004	Germany	winter
4	Alabasskaja	1947	Russia	winter
5	Alba	1987	Poland	winter
6	Albatros Odeskii	1990	Ukraine	winter
7	Alliance	1993	USA	winter
8	Amadeus	1985	Austria	winter
9	Apache	1998	France	winter
10	Arina	1981	Switzerland	winter
11	Arthur 71	1971	USA	winter
12	Atay 85	1985	Turkey	winter
13	Atlas 66*	1948	USA	winter
14	Augusta	1979	USA	winter
15	Aurora	1972	Russia	winter
16	Autonomia	1938	Italy	winter
17	Avalon*	1980	UK	winter
18	Azteca 67	1967	Mexico	winter
19	B16	unregistered	Ukraine	winter
20	Balkan	1979	Serbia	winter
21	Bankuti	1935	Hungary	winter
22	Baranjka	1979	Croatia	winter
23	Begra	1978	Poland	winter
24	Bezostaya	1911	Russia	winter
25	Bilancia	1996	Italy	winter
26	Biscay	2000	Germany	winter
27	Blasco	2002	Italy	winter
28	Ble Des Domes	1940	France	winter
29	Blue A	1950	USA	winter
30	Buck Catriel	1992	Argentina	spring
31	Cadenza*	1992	UK	spring
32	Camp Remy	1980	France	winter
33	Campari*	2003	Germany	winter
34	Caphorn	2004	UK	winter
35	Capo	1989	Austria	winter
36	Cardinal	1986	USA	winter
37	Carmen	1975	Romania	winter
38	Catbird	1991	Mexico	spring
39	CF99007	unregistered	France	winter
40	CF99075	unregistered	France	winter
41	CF99102	unregistered	France	winter
42	CF99105*	unregistered	France	winter
43	Chara	1998	Australia	spring
44	Chinese Spring*	unregistered	China	spring
45	Claire*	1999	UK	winter
46	Courtot	1974	France	winter
47	Crousty*	1994	France	winter

N	Genotype	Release year	Country of origin	Type
48	Cubus	2004	Germany	winter
49	Dekan	1999	Germany	winter
50	Disponent*	1975	Germany	winter
51	Ellvis	2002	Germany	winter
52	Estica*	1990	Netherlands	winter
53	Etoile De Choisy	1950	France	winter
54	Fertodi	1957	Hungary	winter
55	Flamura	1984	Romania	winter
56	Fleischmann	1954	Hungary	winter
57	Frederick	1971	Canada	winter
58	Fundulea	1991	Romania	winter
59	Galahad	1983	UK	winter
60	Gene	1992	USA	winter
61	Gerek 79	1979	Turkey	winter
62	Geronimo	2003	Italy	winter
63	Glenlea	1972	Canada	spring
64	Gloria*	1977	Hungary	winter
65	Granbel	2003	Italy	winter
66	Guarni	2002	Italy	winter
67	Hana	1985	Czech Republic	winter
68	Hereward	1989	UK	winter
69	Herzog*	1986	Germany	winter
70	Iljicovka	1974	Ukraine	winter
71	Isengrain*	1997	France	winter
72	Janz	1989	Australia	spring
73	Kanzler	1980	Germany	winter
74	Karl 92	1992	USA	winter
75	Key	1976	USA	winter
76	Kirac 66	1970	Turkey	winter
77	Kirkpinar 79	1979	Turkey	winter
78	Klein Estrella	1995	Argentina	spring
79	Korweta	1997	Poland	winter
80	Kotuku	1993	New Zealand	winter
81	Krasnodarskaya 99	2003	Russia	winter
82	Kukri	1999	Australia	spring
83	Lasta	1987	Serbia	winter
84	Libellula	1965	Italy	winter
85	Lona	1991	Switzerland	spring
86	Lynx*	1992	UK	winter
87	Magdalena	1949	France	winter
88	Malacca*	1997	UK	winter
89	Manital	1981	Italy	winter
90	Manitoba	1923	Canada	spring
91	Maris Huntsman*	1971	UK	winter
92	Martonvasari 17	1988	Hungary	winter
93	Mexique 50	1950	Mexico	winter
94	Mieti	1992	Italy	winter
95	Milan	1988	Mexico	spring
96	Millenium	2000	USA	winter
97	Momtchil	1982	Bulgaria	winter
98	Monopol	1975	Germany	winter
99	Moulin	1985	UK	winter

N	Genotype	Release year	Country of origin	Type
100	Mv Emese* (reference)	2000	Hungary	winter
101	Mv Palotas	2000	Hungary	winter
102	Mv Suba	2002	Hungary	winter
103	Nap Hal	unregistered	USA	winter
104	Nomade	2003	Italy	winter
105	Ns Rana 1	1975	Serbia	winter
106	Obriy*	1983	Ukraine	winter
107	Ornicar	1997	France	winter
108	Palesio	2000	Italy	winter
109	Pan	1971	Israel	spring
110	Pastor	1993	Mexico	spring
111	Plainsman V	1974	USA	winter
112	Pobeda	1990	Serbia	winter
113	Probstdorfer Perlo	1978	Germany	winter
114	Produttore	1954	Italy	winter
115	Qualital	1991	France	winter
116	Ravenna	1997	Italy	winter
117	Recital	1986	France	winter
118	Red Fife	1842	Canada	spring
119	Red River 68	1968	USA	spring
120	Renan	1989	France	winter
121	Rialto*	1993	UK	winter
122	Riband*	1987	UK	winter
123	Roussalka	1970	Bulgaria	winter
124	Sadovo 1	1972	Bulgaria	winter
125	Sagittorio	1994	Italy	winter
126	San Pastore*	1940	Italy	winter
127	Sarastovskya 29	1957	Russia	spring
128	Sava	1967	Serbia	winter
129	Scout 66	1967	USA	winter
130	Seu Seun 27	1936	Korea	winter
131	Skorospelka 3b	1955	Russia	winter
132	Soissons	1987	France	winter
133	Spark	1991	UK	winter
134	Spartanka*	1988	Russia	winter
135	Stephens	1978	USA	winter
136	Sultan 95	1995	Turkey	winter
137	Sumai 3	1989	China	spring
138	Sunstar	1983	Australia	spring
139	Taldor	1997	France	winter
140	Tam 200	1986	USA	winter
141	Tamaro	1982	Switzerland	winter
142	Thatcher	1934	Canada	spring
143	Thesee	1983	France	winter
144	Tiger*	2001	Germany	winter
145	Tiszataj	1978	Hungary	winter
146	Tommi*	2002	Germany	winter
147	Tremie*	1992	France	winter
148	Ukrainka	1929	Ukraine	winter
149	Valoris*	1998	France	winter
150	Vona	1976	USA	winter
151	Yubileinaya 50	1971	Ukraine	winter

N	Genotype	Release year	Country of origin	Type
152	Yumai 34	1988	China	winter
153	Zvezda	1982	Serbia	winter
154	Mv Makaroni	n.r.	Hungary	durum
155	Parus	1983	Russia	durum
156	Altin	n.r.	Turkey	durum
157	1529-91	germplasm	Bulgaria	durum
158	Orjaune	1995	France	durum
159	Lajtadur	1990	Austria	durum
160	Semperdur	2001	Austria	durum
161	Durabon	1999	Germany	durum
162	Altar 84	n.r.	Mexico	durum
163	Creso	1974	Italy	durum
164	Franckenkorn	1995	Germany	spelt
165	Oberkulmer Rotkorn	n.r.	Switzerland	spelt
166	Spy	n.r.	France	spelt
167	Ressac	n.r.	France	spelt
168	Rouquin	1979	France	spelt
169	Epeautre-Sault De Vaucluse	n.r.	France	Einkorn
170	Mvgb4	germplasm	Hungary	Einkorn
171	08-2004	germplasm	Hungary	Einkorn
172	Mvgb57	germplasm	Hungary	Einkorn
173	122-2004	germplasm	Hungary	Einkorn
174	Mvgb304	germplasm	Hungary	Emmer
175	Mvgb317	germplasm	Hungary	Emmer
176	Mvgb349	germplasm	Hungary	Emmer
177	192-2004	germplasm	Hungary	Emmer
178	265-2004	germplasm	Hungary	Emmer

n.r. not reported